Production of *Sinorhizobium meliloti nod* Gene Activator and Repressor Flavonoids from *Medicago sativa* Roots

José Angelo Silveira Zuanazzi,^{1,2} Pierre Henri Clergeot,¹ Jean-Charles Quirion,² Henri-Philippe Husson,² Adam Kondorosi,^{1,3} and Pascal Ratet¹

¹Institut des Sciences Végétales and ²Institut de Chimie des Substances Naturelles, Centre National de la Recherche Scientifique, Avenue de la Terrasse, F-91198 Gif sur Yvette Cedex, France; and ³Institute of Genetics, Biological Research Center, Hungarian Academy of Sciences, Szeged, P.O. Box 521, H-6701 Hungary

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During symbiosis between leguminous plants and rhizobia, flavonoids exuded by the plants act as chemoattractants and nodulation (*nod*) gene regulators in the other partner. To better understand the role of these compounds during the early steps of the alfalfa-*Sinorhizobium meliloti* symbiosis and the regulation of their production we have isolated *nod* gene inducers from alfalfa roots. All the compounds that we identified in this study as *nod* gene inducers in the root are flavonoids, indicating that other compounds with *nod* gene activator capacity may have little contribution, if any, to *nod* gene activation. Most of the intermediates of the flavonoid pathway were found in *Medicago sativa* roots and nodules, but only end products of the flavonoid pathway were identified in the root exudate. We have also studied flavonoid production in different parts of the root and found that it is developmentally regulated during root growth. Finally, we have shown that coumestrol and medicarpin, present in the exudates and previously described as phytoalexins, possess *nod* gene repressing activity, indicating that the *in vivo nod* gene inducing activity of the root exudate results from positive as well as negative controls of *nod* gene expression by the flavonoids.

Additional keywords: daidzein, 7,4'-dihydroxyflavone, formononetin, isoliquiritigenin, liquiritigenin, 2'-methoxy-isoliquiritigenin.

The establishment of the nitrogen-fixing symbiosis between leguminous plants and the soil bacteria of the family *Rhizobiaceae* leads to the formation of a new organ: the nodule. Inside this nodule, bacteria fix atmospheric nitrogen for the benefit of the plant, which in turn provides carbohydrates necessary for this energy-consuming process (Hirsch 1992; Schultze et al. 1994; Mylona et al. 1995). Recognition be-

tween the two partners takes place at several steps of the process, through molecular signal exchanges. Flavonoids excreted from the roots of leguminous plants activate nodulation (*nod*) gene expression, resulting in the synthesis of Nod factors triggering nodule morphogenesis.

The flavonoids belong to a large group of phenolic compounds (McClure 1975) derived from the phenylpropanoid pathway and are involved in the interaction of the plant with its environment. The flavonoid biosynthetic pathway also leads to the production of related compounds: isoflavonoids, coumestans, tannins, and anthocyanin (Dixon and Paiva 1995; Holton and Cornish 1995). Flavonoids and related compounds fulfill various functions in plants (Shirley 1996). In the leguminous plants, isoflavonoids are phytoalexins and their production is induced in response to pathogen attack (Dixon and Paiva 1995). Molecular studies of this pathway have thus resulted in the characterization of several genes induced during defense responses (Dixon et al. 1992; Esnault et al. 1993). In the rhizosphere of leguminous plants, excreted flavonoids can act as chemoattractants and growth regulators toward pathogenic and symbiotic bacteria (Aguilar et al. 1988; Peters and Verma 1990; Rao 1990; Hartwig et al. 1991; Nair et al. 1991; Dixon and Paiva 1995; Ruan et al. 1995) or as signal molecules required for the induction of bacterial genes involved in pathogenic or symbiotic interactions (reviewed in Peters and Verma 1990; Denarié and Roche 1992; Kondorosi 1992).

In nitrogen-fixing symbiosis the flavonoids are activators of the bacterial regulatory protein NodD. This protein controls the regulation of the *nod* regulon necessary for the synthesis of the bacterial Nod factor acting as a plant morphogen able to trigger nodule morphogenesis (Dénarié and Roche 1992; Kondorosi 1992; Schultze et al. 1994; Fisher and Long 1992). The chemical nature of the flavonoids as well as the NodD sequence are partly responsible for the host specificity of the legume-*Rhizobium* interaction. Each plant produces a different cocktail of flavonoids and only a subset of these compounds is responsible for NodD activation (Firmin et al. 1986; Djordjevic et al. 1987; Rossen et al. 1987; Györgypal et al. 1991). In turn, the chemical nature of the synthesized Nod factor is also responsible for the host specificity of the following steps in the nodule organogenesis (Schultze and Kondorosi 1996).

Corresponding author: Pascal Ratet; Telephone: (1) 69 823 702; Fax: (1) 69 823 695; E-mail: Pascal.Ratet@isv.cnrs-gif.fr

Present address of José Angelo Silveira Zuanazzi: Faculdade de Farmácia, Pontifícia Universidade Católica (RS), Av. Ipiranga, 6681, CEP 90.619-900, Porto Alegre (RS), Brazil.

Flavonoids acting as *nod* gene inducers are present in both roots and seeds, can diffuse in the soil during germination and root growth, and are thus responsible for the formation of a micro-ecosystem favorable for the establishment of the symbiosis (Phillips et al. 1993). In the roots, they are exuded predominantly in the region of growing root hairs (Djordjevic et al. 1987; Peters and Long 1988), the region competent for nodule initiation. The leguminous roots were shown to also exude flavonoids inhibiting *nod* gene expression (Firmin et al. 1986; Djordjevic et al. 1987; Peters and Long 1988), suggesting that the in vivo control of the plant on bacterial *nod* gene expression may result from an antagonistic effect of activating and repressing molecules. However, in these studies the chemical nature of the exuded inhibitors was not identified.

All the flavonoids isolated from alfalfa roots so far are 5-deoxyflavonoids (Phillips et al. 1993; Tiller et al. 1994; Coronado et al. 1995). This chemical structure results from the action of the chalcone reductase (Sallaud et al. 1995). In contrast, in alfalfa seeds and leaves 5-hydroxy-flavonoids including the flavones luteoline and apigenine were found (Phillips et al. 1993; Tiller et al. 1994). Among flavonoids exuded from alfalfa roots, 7,4'-dihydroxyflavone (DHF) and 4,4'-dihydroxy, -2'-methoxychalcone (Phillips et al. 1993; Coronado et al. 1995) are the most potent *nod* gene inducers. Conjugated isoflavonoids and pterocarpanes are also present in large amount in roots (Tiller et al. 1994; Coronado et al. 1995) but do not act as *nod* gene inducers in the alfalfa-*Sinorhizobium meliloti* interaction (Coronado et al. 1995). Their presence (e.g., medicarpin) in the root seems to vary from one analysis to the other (Tiller et al. 1994; Coronado et al. 1995), which may be due to differences in cultivars or in growth conditions. In addition, the betaines trigonelline and stachydrine released from alfalfa seeds were shown to be able to activate the NodD2 protein (Phillips et al. 1992).

We have previously shown that root flavonoid production in alfalfa is nitrogen regulated (Coronado et al. 1995). When the plants were grown under nitrogen limitation, the production of all root flavonoids was increased, accompanied by an enhancement of the *S. meliloti nod* gene inducing activity. This nitrogen-mediated regulation of flavonoid production partially takes place at the transcriptional level.

The aim of the work presented here was to isolate the various (iso)flavonoids present in the roots of the *Medicago sativa* subsp. *varia* cv. A2 and characterize them for their *nod* gene regulating activity. Most of the intermediates of the branched flavonoid pathway starting from the 4,4',2'-hydroxychalcone and ending at the DHF, the malonylononin, and the coumestrol have been identified in root and nodule extracts and their production was localized in the roots of young alfalfa seedlings. In root exudates, however, only DHF and coumestrol were identified, indicating a selective mechanism for their exudation. In addition, we showed that, in contrast to the *nod* gene inducer DHF, the exuded coumestrol specifically acts as a negative regulator of *S. meliloti nod* gene expression.

RESULTS

Rhizobium nod gene inducing activity of the root extracts and exudates is present in the solvent fraction.

We previously showed that the production of *nod* gene inducer flavonoids in *M. sativa* subsp. *varia* cv. A2 roots was

enhanced when plants were grown under limited nitrogen supply (Coronado et al. 1995). In addition, these flavonoids were shown to be nonglycosylated. To determine whether the active compounds present in the root exudates are similar to those of the root extracts, we fractionated both of them in ether, butanol, and aqueous extracts and tested these extracts for their *nod* gene induction capacities with *S. meliloti* strains containing either plasmid pKSK5, carrying the *nodD1* gene, or plasmid pGM108, carrying the *nodD2* gene. In this experiment, all *nod* gene inducing compounds (either flavonoids or other compounds) could theoretically be detected because all fractions (aqueous and solvent) were analyzed. A representative experiment is shown in Figure 1. The use of the Student's *t* test statistical analysis, rather than the comparison of the β -galactosidase units, allowed us to better compare the values obtained with strains overexpressing *nodD1* or *nodD2*. As the *nod* gene inducing activity of the aqueous extract was not statistically different from that of the control, the values are not given here. During the course of the experiment, the level of *nod* gene induction was significantly higher in all the root samples (extract and exudates) of plants grown under limited nitrogen supply (Fig. 1) than in those grown under nonlimited nitrogen supply, irrespective of the activator NodD1 and NodD2 regulatory proteins, confirming our previous results (Coronado et al. 1995). The experiment also showed that the *nod* gene inducing activity was higher in root extracts than in exudates.

This analysis indicated that in the roots and exudates of plants grown under limited nitrogen supply, the production of the *nod* gene inducing metabolites was increased. Moreover, the level of *nod* gene inducing activity was higher in the ether extract than in the butanol extract (Fig. 1), confirming also that the active flavonoid compounds were present both in the roots and exudates in the nonglycosylated form (Coronado et al. 1995).

Fig. 1. *Rhizobium nod* gene inducing activity of extracts (ether and butanol) prepared from roots and exudates of *Medicago sativa* subsp. *varia* cv. A2 grown under nonlimited and limited combined nitrogen. *M. sativa* subsp. *varia* cv. A2 plants were grown in nonlimited (10 mM KNO₃; 10) nitrogen solution or in limited (0.25 mM KNO₃; 0.25) nitrogen solution. Extracts were used to induce a *nodC::lacZ* gene fusion in derivatives of *Sinorhizobium meliloti* JM57 containing either plasmid pKSK5 carrying the *nodD1* gene or plasmid pGM108 carrying the *nodD2* gene. β -Galactosidase activity is expressed in Student's *t* test values after statistical analysis. All assays were performed at least three times with 10 samples in each experiment (GL = 18).

Detection of only flavonoids as *nod* gene inducers in the ether extract.

Preliminary analytical high-pressure liquid chromatography (HPLC) revealed the presence of several compounds in the active fraction of the exudate. However, the amount of products in this fraction (24 mg) was not sufficient to allow further preparative chromatography and spectral analyses. Thus, detailed studies on the root extract were made by assuming that the products identified in the roots would also be present in the exudate. For the identification of the compounds responsible for *nod* gene induction, we used the ether extract of the plants grown under limited nitrogen supply, as this extract contained the strongest *nod* gene inducing activity (Fig. 1). Characterization of the butanol extract, containing mainly the isoflavonoid conjugates ononin and malonylononin, was described previously (Coronado et al. 1995).

The material extracted by ether (2.5 g) was chromatographed on a Vacuum Liquid Chromatography column (Coll and Bowden 1986) over silica gel (35 g) with solvents of increasing polarity and petroleum ether, CH₂Cl₂, and MeOH. Eight fractions, 250 ml each, were collected and analysis of flavonoid content was performed by testing them for *nod* gene induction capacity (by measuring β -galactosidase activity) and by analytical HPLC. Only the third fraction showed significant *nod* gene inducing activity (data not shown) and, therefore, was chromatographed by preparative HPLC (system A). With this technique, 11 fractions were prepared and analyzed for their *nod* gene inducing activity and by analytical HPLC. From these 11 new fractions only the first four significantly induced the expression of *nod* genes. In addition, the fifth fraction contained a pure flavonoid and was further analyzed. Eight flavonoids were isolated from the first four fractions by new preparative HPLC (system B). The characteristics of the flavonoids present in each fraction and shown in Figure 2 are as follows.

Fraction 1.

In the first fraction a flavonoid (2 mg) was isolated and after analysis of these spectral data, (shown below) the structure of the liquiritigenin (7,4'-dihydroxyflavanone) **{1}**, was proposed.

{1}: UV: $\lambda_{\max}$ (nm) – (MeOH): 207, 228 (sh), 276 and 312 (sh). After the addition of NaOMe: $\lambda_{\max}$ (nm): 207, 250, 290 (sh), 326 (sh), 335.

Electronic impact mass spectra (EIMS): m/z (%): 256 [M]⁺• (100), 255 [M]⁺•-H⁺ (50), 163 [M]⁺•-B (31), 137 [A₁+H]⁺ (93), 136 A₁⁺• (7), 120 B₃⁺• (43), 108 A₁⁺•-CO (7), 107 (17).

¹H nuclear magnetic resonance (NMR) (CD₃OD) δ (ppm): 2.79 (1H, *dd*, J = 16.9 and 3.0 Hz, H-3 *cis*), 3.15 (1H, *dd*, J = 16.9 and 13.0 Hz, H-3 *trans*), 5.47 (1H, *dd*, J = 13.0 and 3.0 Hz, H-2), 6.46 (1H, *d*, J = 2.2 Hz, H-8), 6.59 (1H, *dd*, J = 8.7 and 2.2 Hz, H-6), 6.91 (2H, *d*, J = 8.6 Hz, H-3' and H-5'), 7.41 (2H, *d*, J = 8.6 Hz, H-2' and H-6'), 7.81 (1H, *d*, J = 8.7 Hz, H-5).

Fraction 2.

The second fraction was separated in three new subfractions by preparative HPLC (system B). In this first subfraction less than 1 mg of compound was isolated. After UV profile analysis of the peak by analytical HPLC system (photodiode array [PDA] detector; at 200 to 400 nm), the structure of the isofla-

vonoid daidzein (7,4'-dihydroxyisoflavone) **{2}** was deduced and confirmed by co-injection with a standard in analytical HPLC.

The second subfraction (1 mg) was not completely pure. Further purification was not possible but we could obtain the spectral data and the combination of the results allowed the proposition of the structure of the 4,4'-dihydroxy-2'-methoxy chalcone (2'-methoxy isoliquiritigenin) **{3}**. These spectral data are as follows.

{3}: UV: $\lambda_{\max}$ (nm) – (MeOH): 205, 280 (sh) and 345 (note: the $\lambda_{\max}$ values between 200 and 300 nm were hidden by the impurities). The UV spectra made by the peak in HPLC (PDA detector) by the standard and sample were as follows: $\lambda_{\max}$ (nm): 200, 236, 352 and 200, 237, 352, respectively.

EIMS: m/z (%): 270 [M]⁺• (55), 269 [M]⁺•-H⁺ (24), 255 [M]⁺•-Me (33), 242 [M]⁺•-CO (36), 165 (15), 164 (61), 163 [M]⁺•-B (27), 152 (24), 151 A₂⁺ (100), 147 B₅⁺ (39), 137 [A₁+H]⁺ (25), 121 (58), 120 B₃⁺• (24), 119 [B₅ - CO]⁺ (45), 108 [A₁-CO]⁺ (36), 107 (64).

¹H NMR (CD₃OD) δ (ppm): 3.9 (3H, *s*, OMe), 6.3 – 6.4 (2H, *m*, H-3' and H-5'), 6.9 (2H, *d*, J = 8.6 Hz, H-3 and H-5), 7.4 – 7.8 (5H, *m*, H-6', H β , H-2, H-6 and H α).

In the third subfraction (2 mg) after the spectral analysis the structure of the DHF was proposed **{4}**. The spectral data are as follows.

Fig. 2. Flavonoids identified in the *Medicago sativa* subsp. *varia* cv. A2 root and exudate extracts (ether), grown under limited combined nitrogen supply. Flavonoids are placed following their position in the flavonoid biosynthetic pathway. 1: liquiritigenin, 2: daidzein, 3: 2'-methoxyisoflavonoid, 4: 4,4'-dihydroxyflavanone (DHF), 5: 2'-hydroxyformononetin, 6: isoliquiritigenin, 7: coumestrol, 8: formononetin.

UV (MeOH): 205, 230 (sh), 247 (sh), 310 (sh) and 327.

EIMS: m/z (%): 254 $[M]^{+•}$ (100), 253 $[M]^{+•}-H^{•}$ (30), 226 $[M]^{+•}-CO$ (33), 137 $[A_1+H]^+$ (58), 118 $B_1^{+•}$ (33).

1H NMR (CD_3OD) δ (ppm): 6.78 (1H , s , H-3), 7.03 (1H , dd , $J = 8.7$ and 2.2 Hz, H-6), 7.04 (2H , d , $J = 8.6$ Hz, H-3' and H-5'), 7.08 (1H , d , $J = 2.2$ Hz, H-8), 7.98 (2H , d , $J = 8.6$, H-2' and H-6'), 8.07 (1H , d , $J = 8.7$ Hz, H-5).

Fraction 3.

This fraction was a mixture of two flavonoids, and was chromatographed further to give two new subfractions. The first one (2 mg) contained the DHF **{4}**, already identified in the second fraction. In the second subfraction (1 mg) we identified the 2'OH-formononetin **{5}**, also present in fraction 4.

Fraction 4.

After preparative HPLC, three new subfractions were separated. In the first one (3 mg), we identified the 7,2'-dihydroxy-4'-methoxyisoflavone (2'OH-formononetin) **{5}**. The corresponding spectral data are as follows.

{5}: UV: λ_{max} (nm) – (MeOH): 207, 248 (sh), 260 (sh), 284 (sh), and 304 (sh). After addition of NaOMe λ_{max} (nm): 206, 256, and 335.

EIMS: m/z (%): 284 $[M]^{+•}$ (100), 283 $[M]^{+•}-H^{•}$ (14), 267 $[M]^{+•}-OH$ (34), 151 (43), 148 $B_1^{+•}$ (73), 142 (8), 137 $[A_1+H]^+$ (26), 133 (16).

1H NMR (CD_3OD) δ (ppm): 3.85 (3H , s , OMe), 6.6 (2H , m , H-3' and H-5'), 6.97 (1H , d , $J = 2.2$ Hz, H-8), 7.05 (1H , dd , $J = 8.9$ and 2.2 Hz, H-6), 7.24 (1H , d , $J = 8.9$ Hz, H-6'), 8.16 (1H , d , $J = 8.9$ Hz, H-5), 8.23 (1H , s , H-2).

^{13}C NMR (CD_3OD) δ (ppm): 55.7 (-OMe), 103.2 (C-3' and C-8), 106.6 (C-5'), 116.7 (C-6), 117.9 (C-10), 121.5 (C-1'), 124.0 (C-3), 128.5 (C-5), 133.0 (C-6'), 156.6 (C-2), 157.9 (C-2'), 160.0 (C-4'), 162.6 (C-9), 165.1 (C-7).

In the second subfraction, we identified the 4,4',2'-trihydroxychalcone (isoliquiritigenin) **{6}**. The spectral data are as follows.

{6}: UV: λ_{max} (nm) – (MeOH): 205, 230 (sh), 308 (sh) and 363. After addition of NaOMe: 203, 244 (sh), 272 (sh), and 418. After addition of $AlCl_3$: 203, 330 (sh), 370 (sh), and 417.

EIMS, m/z (%): 256 $[M]^{+•}$ (83), 255 $[M]^{+•}-H^{•}$ (42), 239 $[M]^{+•}-OH$ (11), 228 $[M]^{+•}-CO$ (8), 163 $[M]^{+•}-B$ (26), 147 $B_5^{+•}$ (11), 137 $A_2^{+•}$ (100), 120 $B_3^{+•}$ (67).

1H NMR (CD_3OD) δ (ppm): 6.37 (1H , d , $J = 2.2$ Hz, H-3'), 6.51 (1H , dd , $J = 8.9$ and 2.2 Hz, H-5'), 6.93 (2H , d , $J = 8.8$ Hz, H-3, and H-5), 7.71 (1H , d , $J = 15.3$ Hz, H- α), 7.72 (2H , d , $J = 8.8$ Hz, H-2 and H-6), 7.89 (1H , d , $J = 15.3$ Hz, H- β), 8.07 (1H , d , $J = 8.9$ Hz, H-6').

^{13}C NMR (CD_3OD) δ (ppm): 104 (C-3'), 109 (C-5'), 114 (C-1'), 117 (C-3 and C-5), 118 (C- α), 127 (C-1), 132 (C-2 and C-6), 133 (C-6'), 145 (C- β), 160 (C-4) 167 (C-4') 192 (C- β').

In the third subfraction we identified the 3,9-dihydrocoumestan (coumestrol) **{7}**. The spectral data of this compound are as follows.

{7}: UV: λ_{max} (nm) – (MeOH): 206, 241, 254 (sh), 302, 342, and 356 (sh). After addition of NaOMe, λ_{max} (nm): 206, 244 (sh), 273, 310, and 378.

EIMS: m/z (%): 268 $[M]^{+•}$ (100), 240 $[M]^{+•}-CO$ (8), 212 – CO (3), 184 – CO (3), 155 (3), 134 (3).

1H NMR (CD_3OD) δ (ppm): 6.98 (1H , d , $J = 2.2$ Hz, H-5'), 7.01 (1H , dd , $J = 8.6$ and 2.2 Hz, H-6), 7.02 (1H , dd , $J = 8.4$

and 2.2 Hz, H-3'), 7.19 (1H , d , $J = 2.2$ Hz, H-8), 7.85 (1H , d , $J = 8.4$ Hz, H-2'), 7.96 (1H , d , $J = 8.6$ Hz, H-5).

^{13}C NMR (CD_3OD) δ (ppm): 99.5 (C-5'), 104.2 (C-8), 114.9 (C-3), 115.1 (C-3'), 115.5 (C-6), 116.5 (C-10), 123.7 (C-1'), 122.1 (C-2'), 128.7 (C-5), 158.0 (C-9), 158.6 (C-7), 163.4 (C-4').

Fraction 5.

This last fraction was not able to induce *nod* gene expression, but it was the most abundant one (41 mg) and contained only one flavonoid. This flavonoid was identified as the 7-hydroxy-4'-methoxyisoflavone (formononetin) **{8}**, by means of its spectral data:

{8}: UV: λ_{max} (nm) – (MeOH): 205, 236 (sh), 248, 260 (sh), and 302. After addition of NaOMe, λ_{max} (nm): 205, 254, 308, and 326.

EIMS: m/z (%): 268 $[M]^{+•}$ (100), 267 $[M]^{+•}-H^{•}$ (33), 253 $[M]^{+•}-Me$ (21), 136 $A_1^{+•}$ (11), 134 $[M]^{++}$ (18), 132 $B_1^{+•}$ (80), 108 $A_1^{+•}-CO$ (15).

1H NMR (CD_3OD) δ (ppm): 3.85 (3H , s , OMe), 6.91 (1H , d , $J = 2.2$ Hz, H-8), 6.98 (1H , dd , $J = 8.7$ and 2.2 Hz, H-6), 7.03 (2H , dd , $J = 8.8$ and 2.1 Hz, H-2' and H-6'), 7.55 (2H , dd , $J = 8.8$ and 2.1 Hz, H-3' and H-5'), 8.10 (1H , d , $J = 8.7$ Hz, H-5), 8.27 (1H , s , H-2).

The structures of the isolated flavonoids were confirmed by co-injection with standards in analytical HPLC and spectral data (EIMS, UV, and NMR). All flavonoids identified were already described in alfalfa or other plants and their spectral data are comparable with data in the literature (Mabry and Markham 1975; Markham and Mabry 1975; Harborne 1988; Phillips et al. 1993). The position of the compounds whose pics can be unambiguously detected is indicated on the chromatogram shown in Figure 3A.

The *nod* gene inducing activities of the isoliquiritigenin, 2'-methoxy isoliquiritigenin, liquiritigenin, and DHF were already described (Phillips et al. 1993) and were confirmed in our experiments (data not shown). All other compounds identified here are isoflavonoids and do not possess any *nod* gene inducing activity (data not shown). However, as described below we found that some of them may act as negative regulators of *nod* gene expression.

Some root flavonoids are also present in the exudate.

The presence of the eight flavonoids identified in the roots was looked for in the exudates by analytical HPLC with co-injection of standards against the ether extract. Among them, only the DHF **{4}** and the coumestrol **{7}** were identified (Fig. 3B) in the exudate of the plants grown under limited nitrogen supply. In the exudate of the plants grown under nonlimited nitrogen supply, these two compounds were below the detection level (with the same HPLC conditions), indicating that their production was reduced in this exudate, in agreement with the *nod* gene inducing activity of this extract (Fig. 1). Formononetin, the most abundant flavonoid present in the root extract, was not detected in the exudate. Medicarpin, a phytoalexin commonly found in other alfalfa cultivars (Dixon et al. 1992; Tiller et al. 1994) but not found among the flavonoids present in the root extract of cultivar A2, was also searched for by analytical HPLC in the ether extracts of roots and exudates. As we have previously shown, this compound was not detected in the root extract (Coronado et al. 1995) but

trace amounts were found in the exudates of plants grown under limited nitrogen supply (data not shown).

Nodule flavonoids are not significantly different from root flavonoids.

The presence of the flavonoids detected in root extracts was also looked for in butanolic and ether extracts of nodules by analytical HPLC with co-injection of standards. The HPLC profile of these extracts was not significantly different from that of the corresponding root extracts (data not shown). However, as indicated in Table 1, the amount of the identified compounds was reduced in the butanol and ether extracts from nodules, compared with the corresponding root extracts of

nodulating plants or of noninoculated plants grown under limited nitrogen supply. These results also indicate that the respective proportion of the different compounds remained relatively similar in the two organs, formononetin being the most abundant aglycone in the three ether extracts. The presence of new flavonoids was also searched for in the nodule extracts, but new flavonoid compounds could not be identified in these extracts. This result thus indicates that the major flavonoids in nodules and roots are similar.

nod gene inducing activity of the exuded flavonoids.

The bacterial *nod* gene regulators are supposed to interact with the flavonoids released into the rhizosphere, rather than with the flavonoids present in the roots. Thus, the *nod* gene regulating activities of the DHF, coumestrol, and medicarpin identified in the exudates were analyzed. As previously described (Phillips et al. 1993; Coronado et al. 1995), DHF was a potent *nod* gene inducer (Table 2) when used in combination with the *S. meliloti* NodD1 (*RmNodD1*) and NodD2 (*RmNodD2*) regulatory proteins. Interestingly, neither of the phytoalexins coumestrol or medicarpin acts alone as *nod* gene inducer but, when tested in conjunction with DHF, they could inhibit its inducing activity. Increasing the inducer/inhibitor ratio resulted in a reduction of this inhibition (Table 2), indicating that these compounds may act as competitive inhibitors when used in combination with the *RmNodD1* and *RmNodD2* regulatory proteins. As indicated in Table 2, similar results were obtained with luteolin or the chalcone isoliquiritigenin (Fig. 2). With these two flavonoids, coumestrol could also act as a *nod* gene inhibitor, in combination with both regulatory proteins, even if their *nod* gene inducing activity was stronger when used in combination with *RmNodD1* (Table 2).

To confirm that the negative effects of the phytoalexins were not due to a more general metabolic effect or only to competition for flavonoid binding sites present at the bacterial surface (Hubac et al. 1994), the *nod* gene inducing activity of

Fig. 3. High-pressure liquid chromatography (HPLC) analysis between 250 and 400 nm of *Medicago sativa* subsp. *varia* cv. A2 root extract and exudate. Samples were analyzed at 10 mg/ml. **A**, HPLC chromatogram of a root ether extract. Positions of flavonoids identified in this work are indicated. O: ononin, 1: liquiritigenin, 4: 7,4'-dihydroxyflavone (DHF), 5: 2'-hydroxyformononetin, 6: isoliquiritigenin, 7: coumestrol, 8: formononetin. **B**, HPLC chromatogram of an exudate extract. Positions of DHF (4) and coumestrol (7) are indicated on the chromatogram.

Table 1. Flavonoid content of roots and nodules^a

Plan material	Extract ^b					
	Butanol	Ether				
		Flavonoid				
	GlycF ^c	Form ^d	Coum ^d	Iso ^d	20HF ^d	DHF ^d
Root N ^e	355	600	20	10	9	9
Nod-root ^f	233	430	30	15	10	25
Nod ^g	121	40	5	3	<1	1

^a Values given are arbitrary, represent amounts of the different flavonoids in the butanol and ether extracts, and were estimated by measuring the surface of the peaks in the high-pressure liquid chromatography (HPLC) chromatograms. Values do not represent the exact amount of each compound because the UV absorbance of each flavonoid is different, but they allow comparison of the amounts of flavonoids present in the different extracts.

^b Amount of flavonoids in the butanol and ether extracts cannot be compared. Each extract was analyzed by injecting 10 µl of extract at a concentration of 10 mg/ml in ethanol:dimethyl sulfoxide (95:5, vol/vol).

^c Value for glycosylated formononetin (GlycF) represents sum of the values obtained for ononin and malonyl-ononin.

^d Formononetin (Form); coumestrol (coum); isoliquiritigenin (iso); 2'-hydroxyformononetin (20HF); 7,4'-dihydroxyflavone (DHF).

^e Root of plants grown under limited nitrogen supply.

^f Root of nodulated plants.

^g Nodules.

DHF and coumestrol was also tested in strain JM57(pBH264) harboring the *nodD1* regulatory gene of the broad host range *Rhizobium* sp. MPIK3030 (MPIK-NodD1) (Györgypal et al. 1988). The result of this experiment (Table 3) showed that both DHF and coumestrol acted as strong *nod* gene inducers in this strain, DHF being a stronger inducer than coumestrol. Moreover DHF was 1,000-fold more active as *nod* gene inducer in *S. meliloti*, when used in combination with the MPIK-NodD1, instead of the *RmNodD1* and *RmNodD2* regulatory proteins. In the presence of the MPIK-NodD1 regulatory protein, the action of the two compounds was additive instead of being antagonistic, indicating that the negative effects observed in conjunction with *RmNodD1* or *RmNodD2* were specific for these two regulatory proteins, and were not due to a more general negative physiological effect. Similar results were described by Györgypal et al. (1991) with the phytoalexin biochanine A in combination with this regulatory protein, but this compound was not found in alfalfa. In our assay conditions, the growth of the bacteria was not affected during the experiment, indicating that neither coumestrol nor medicarpin act as a phytoalexin toward the symbiotic partner, as also previously reported by Pankhurst and Biggs (1980).

Localization of flavonoid production in the root.

Using, respectively, *R. leguminosarum* bv. *trifolii* and *S. meliloti* derivatives containing *nodC::lacZ* gene fusions as indicator, Djordjevic et al. (1987) and Peters and Long (1988) showed that root *nod* gene inducer production was localized to the part of the root where root hairs were developing. This phenomenon could result from either an increased local production of inducing flavonoids or from an increased exudation of these compounds, or from both. To try to unravel the mechanism controlling root flavonoid release, we dissected

290 alfalfa root seedlings 48 h after germination in three parts that differed by their *nod* gene inducing capacities, and their flavonoid contents were analyzed. Localization of the *nod* gene inducing flavonoids in the root was achieved with a *nodC::lacZ* gene fusion containing *S. meliloti* (Rm41 carrying pRm57). The different regions of the root defined in this way were the following: (i) the root tip, without *nod* gene inducing activity on petri dishes; (ii) the root hair growing zone, excreting *nod* gene inducers; and (iii) the older part of the root lacking this inducing activity (data not shown). This pattern of *nod* gene induction detected in vivo was identical to that previously reported by Djordjevic et al. (1987) and Peters and Long (1988). At this age no lateral root formation occurred. When the seedlings were tested 2 days later, *nod* gene inducing zones appeared at sites where lateral roots emerged from the main root, probably revealing local wounding resulting from the development of the lateral root meristem, which is in agreement with the detection of *chs* transcripts in these regions (Yang et al. 1992).

Flavonoids extracted from these three parts, called fraction F1 for flavonoids extracted from the root tip, fraction F2 for flavonoids extracted from the root hair growing zone, and fraction F3 for flavonoids extracted from the mature part of the root, were analyzed by HPLC. This analysis (Fig. 4A,B,C; analysis at 270 nm, detecting mostly the isoflavonoids) indicated that all fractions contained ononin and malonylononin as the major flavonoids. In addition, the root region corresponding to the zone of maximum *nod* gene inducing activity (F2, Fig. 4B) contained the highest amount of flavonoids (note that the scale of the chromatogram of Figure 4B is different) when compared with fractions F1 and F3 (Fig. 4A,C). This indicates that the increased flavonoid content of this fraction was not restricted to the *nod* gene inducers, as the production of the major flavonoids (ononin and malonylononin) was also increased. In contrast, the production of their precursor, the formononetin, did not exhibit any significant increase in this fraction and was comparable in the three extracts. Moreover, in each fraction other compounds (X,Y,Z) were detected (Fig. 4), but their nature could not be determined due to the limited amount of material obtained. Only compound Z was tentatively identified as a medicarpin derivative (medicarpin-7-*O*-glucose or its malonyl derivative), by its UV spectrum (data not shown) and retention time in fraction F3. This compound has already been described in alfalfa root extracts (Tiller et al. 1994) and its absence in the *M. sativa* subsp. *varia* root extract (see above) may be explained by possible cultivar differences. Analysis of these fractions at 330 nm (Fig. 4D) allowed de-

Table 2. Positive and negative control of *nod* gene expression in *Sinorhizobium meliloti* mediated by *RmNodD1* and *RmNodD2* regulatory proteins^a

Flavonoid	<i>RmNodD1</i>	<i>RmNodD2</i>
DHF 2 µM	21.6 (± 3.13)	28.2 (± 4.36)
DHF 4 µM	35.8 (± 5.6)	37.8 (± 2.2)
DHF 8 µM	63.5 (± 13.0)	54.6 (± 5.7)
coum 1 µM	0.4 (± 0.3)	1.0 (± 1.78)
coum 10 µM	0	0
med 10 µM	0	0
DHF 2 µM + coum 1 µM	23.4 (± 3.1)	23.3 (± 3.0)
DHF 2 µM + coum 5 µM	7.0 (± 1.9)	6.9 (± 1.9)
DHF 2 µM + coum 10 µM	6.7 (± 0.9)	4.1 (± 1.3)
DHF 2 µM + med 10 µM	3.1 (± 0.5)	5.3 (± 0.6)
DHF 4 µM + coum 4 µM	45.6 (± 6.1)	26.9 (± 2.7)
DHF 8 µM + coum 8 µM	75.1 (± 10.1)	39.3 (± 2.3)
lut 10 µM	90.4 (± 6.9)	8.8 (± 1.5)
iso 1 µM	67.5 (± 7.5)	21.6 (± 3.3)
lut 10 µM + coum 10 µM	65.0 (± 5.2)	1.0 (± 2.2)
iso 1 µM + coum 10 µM	18.2 (± 1.7)	0.9 (± 1.6)

^a *nod* gene inducing activity of the compounds was tested in strain JM57 carrying the *nodC::lacZ* gene fusion integrated in the megaplasmid and containing either plasmid pKSK5 carrying the *S. meliloti nodD1* gene (*RmNodD1*) or plasmid pGM108 carrying the *S. meliloti nodD2* gene (*RmNodD2*). β-Galactosidase is expressed in Miller units. Value of the uninduced controls (7 and 12 Miller units for *RmNodD1* and *RmNodD2*, respectively) was subtracted from each value. Standard deviation values are indicated for each value. Abbreviations: DHF: 7,4'-dihydroxyflavone; coum: coumestrol; med: medicarpin; lut: luteolin; and iso: isoliquiritigenin.

Table 3. *nod* gene inducing activity of 7,4'-dihydroxyflavone (DHF) and coumestrol (coum) in *Sinorhizobium meliloti* mediated by the MPIK-NodD1 regulatory protein^a

Flavonoid	MPIK-NodD1
DHF 5 nM	76 (± 2)
DHF 10 nM	211 (± 12)
coum 50 nM	98 (± 13)
coum 100 nM	304 (± 5)
DHF 5 nM + coum 50 nM	248 (± 13)

^a *nod* gene inducing activity of the compounds was tested in strain JM57 containing plasmid pBH264 carrying the *Rhizobium* sp. MPIK3030 *nodD1* (MPIK-NodD1). β-Galactosidase is expressed in Miller units. Value of the uninduced control (29 Miller units) was subtracted from each value. Standard deviation values are indicated for each value.

tection of the presence of minor flavonoids including the *nod* gene inducers methoxychalcone (MeCh), DHF, and isoliquiritigenin. The production of this latter compound, the direct product of CHS activity and thus the precursor of all root flavonoids, did not seem to vary between fractions F1 and F2. In

contrast, the production of MeCh and DHF (both are strong *nod* gene inducers) as well as the isoflavonoid daidzein and the *nod* gene repressor coumestrol, increased in fraction F2.

To correlate these changes in the amount of flavonoids seen in the HPLC profiles with the *nod* gene inducing activity of

Fig. 4. Localization of flavonoids in different parts of alfalfa seedling roots. Extracts were analyzed at a concentration of 25 mg/ml. **A**, Fraction F1, root tip. **B**, Fraction F2, root hair growing zone. **C**, Fraction F3, mature part of the root. All were analyzed at 270 nm. **D**, Comparison of the high-pressure liquid chromatography (HPLC) profile of fractions F1, F2, and F3 analyzed at 330 nm. F1: solid line; F2: dotted line; F3: dashed line. The scale of **B** has been changed as a result of increased content in flavonoids of this fraction. O: ononin, MO: malonylononin, 2: daidzein, 3: 2'-methoxy isoliquiritigenin, 4: 7,4'-dihydroxyflavone (DHF), 6: isoliquiritigenin, 7: coumestrol, 8: formononetin., X, Y, Z: unidentified compounds (see Results section).

exudates from the different root zones detected in vivo, the *nod* gene inducing activities of fractions F2 and F3 were tested in conjunction with *RmNodD1* and *RmNodD2* regulatory proteins, respectively. As the *nod* gene inducing activities of these fractions turned out to be low and were mainly present in the solvent fraction of root extracts (see Figure 2), these fractions were extracted with ether to separate glycosylated and nonglycosylated flavonoids, then the ether extract was redissolved in a volume proportional to the volume of the fraction before extraction. Because these new fractions were very small, the amount of material in them could not be determined. However, with this approach, *nod* gene inducing activity could be detected in the ether extract of fraction F2 but not in that of fraction F3 (data not shown), in agreement with the amount of flavonoids that could be observed in these two fractions (Fig. 4) by HPLC. Thus, the amount of flavonoids exuded from the root in the zone of growing root hairs is proportional to the amount of flavonoids accumulating in this region of the root.

DISCUSSION

In this paper an extensive analysis of root compounds acting as *nod* gene regulators in *M. sativa* subsp. *varia* cv. A2 is presented. These experiments showed that the *nod* gene inducers detected in the root extract were represented exclusively by flavonoids. No other compounds, like betaines (Phillips et al. 1992) found in alfalfa seed exudates, were identified in this work, indicating that their production might be restricted to the seeds or they are present in the root in an amount not significant for *nod* gene activation. In fact, if compounds like betaines were present in the roots of our plants, *nod* gene inducing activity could be detectable in the aqueous extract.

We have identified all the flavonoids of the biosynthetic pathway starting with isoliquiritigenin (4,4'-dihydroxychalcone), as well as the *nod* gene activator DHF and the different intermediates of the isoflavonoid pathway, except vestitone. Most of these compounds have already been described in alfalfa, but to our knowledge such a complete analysis of the flavonoid pathway in one plant was not reported previously. In addition, 2'-hydroxyformononetin has never been reported for alfalfa, but was isolated for the first time from white clover (Woodward 1981). 2'-OMe isoliquiritigenin was first isolated from *Pisum sativum* (Carlson and Dolphin 1982) and described in alfalfa as a strong *nod* gene inducer by Maxwell et al. (1989). Isoliquiritigenin was identified in alfalfa leaves by Ingham (1979). DHF was identified the first time in alfalfa and clover by Bickoff et al. (1965). Its production was shown to be increased in leaves after treatment with ozone and infection by pathogenic fungi or bacteria (Hurwitz et al. 1979; Skärby and Pell 1979; Sallaud et al. 1997). Daidzein was identified in alfalfa leaves by Guggolz et al. (1961). In the cultivar used for our study (*M. sativa* subsp. *varia* cv. A2) the phytoalexin coumestrol was found to be the end product of the isoflavonoid pathway, instead of the medicarpin isolated in previous studies (Dixon and Paiva 1995). Coumestrol was previously found in alfalfa leaves after treatment with pathogenic fungi (Bickoff et al. 1957; Francis and Millington 1971; Olah and Sherwood 1971). Tiller et al. (1994) have also identified coumestrol in alfalfa roots but mainly in malonyl gluco-

side conjugated form. We were not able to detect conjugated coumestrol in our extracts (Coronado et al. 1995). These differences may reflect cultivar differences or differences in growth conditions. In addition, we show here that the different flavonoids identified in roots could also be found in nodules, albeit in reduced quantities, and that no other type of flavonoids could be identified in this organ by analytical HPLC. Thus, this analysis suggests that the most abundant flavonoids are identical in the two organs.

We have previously shown that the production of these compounds was regulated by the nitrogen nutritional status of the plant (Coronado et al. 1995). In addition, it was previously shown that production of *nod* gene inducing flavonoids was restricted to the zone of emerging root hairs (Djordjevic et al. 1987; Peters and Long 1988). Our analysis of the flavonoid content from different parts of the root showed that this increase in *nod* gene induction around the emerging root hair zone correlated with a general increase in flavonoid production in this part of the root because the production of compounds unable to act as *nod* gene activators (glycosylated formononetin derivatives) as well as the production of the *nod* gene repressor coumestrol were also enhanced in this region. Though our experiment did not reveal a specific increase in flavonoid exudation at this region of the root, our results indicate that the increased *nod* gene induction partially resulted from an increase in flavonoid production.

Several authors have shown that pea root tips contain high amounts of *chs* transcripts (Yang et al. 1992; McKhann and Hirsch 1994) and CHS protein (Rommewinkel et al. 1992). This observation did not correlate with the production of high amounts of flavonoids in this region of the root in alfalfa, or the type of flavonoids produced in this area was not detectable in our HPLC analyses or in the *nod* gene inducing experiments (Djordjevic et al. 1987; Peters and Long 1988; our results). Another possibility is that the CHS synthesized in the apical part of the root is active only when cells differentiate in the emerging root hair zone. However, in these localization experiments the emerging root hair zone was not analyzed and therefore might have contained even higher amounts of the CHS protein. Recently, McKhann et al. (1997) localized *chs* transcript accumulation in alfalfa root hairs following *S. meliloti* infection, indicating that these flavonoids are produced in this region, in agreement with our experiments (Fig. 4) and previous reports (Djordjevic et al. 1987; Peters and Long 1988). All these results indicate that flavonoid production is developmentally regulated during root growth.

We show here that exudation of flavonoids is not likely to be a passive mechanism, because only a subset of the compounds isolated from the root extract was present in the exudate. Interestingly, both exuded compounds (DHF and coumestrol) represent nonglycosylated end products of the flavonoid pathway. Thus, a specific secretion mechanism may exist for these two compounds, or the intermediates of the pathway are in a cell compartment that does not allow their exudation.

Analysis of the *nod* gene inducing activity of the exuded compounds revealed that coumestrol and medicarpin have antagonistic effects on the *nod* gene inducing activity of several flavonoids, when used in combination with the *S. meliloti* NodD regulatory proteins. DHF, luteolin, and isoliquiritigenin are potent *nod* gene inducers (see also Phillips et al. 1993; Coronado et al. 1995) in conjunction with the NodD1 or

NodD2 proteins. In contrast, coumestrol acts as a *nod* gene repressor with these proteins. DHF and coumestrol activated *nod* gene expression with the NodD1 protein of the broad host range *Rhizobium* sp. MPIK3030 (Györgypal et al. 1988) in the same genetic background (*S. meliloti* JM57), indicating that the negative regulatory effect of this latter compound on *RmNodD1* and *RmNodD2* activity is specific for these two regulatory proteins. Thus, both compounds interact with the narrow as well as the broad host range regulatory proteins, indicating that their host range characteristics do not depend on their binding capacity to the regulatory proteins but rather on their capacity to activate these proteins for *nod* gene induction. It is interesting to note that *nod* gene expression resulting in Nod factor production must be maintained at a low level in order to allow optimal nodulation of alfalfa by *S. meliloti* (Kondorosi 1992). This low level of Nod factor production might be necessary to avoid elicitation of a plant defense response by the Nod factor at high concentration (Savouré et al. 1997). Thus, it seems that the negative regulation of *S. meliloti nod* gene expression by some flavonoids may contribute to an optimal level of *nod* gene expression. These studies also show that the measured *nod* gene inducing activity of the root extracts or exudates is determined not simply by the *nod* gene inducing flavonoids but rather by the overall action of both inducing and repressing flavonoids.

MATERIALS AND METHODS

Plant material.

Root flavonoids were isolated and identified from the alfalfa cultivar *M. sativa* subsp. *varia* cv. A2 (Deak et al. 1986). Flavonoid localization experiments were performed with *M. sativa* subsp. *sativa* cv. Sital as previously described (Bauer et al. 1994).

Plant growth.

Plants were grown as described previously (Coronado et al. 1995). The complete root system and root exudates were harvested after 15 days of growth in the nutrient solution described by Blondon (1964), supplemented with either 0.25 mM KNO₃ (limited nitrogen supply [N⁻]) or 10 mM KNO₃ (nonlimited nitrogen supply [N⁺]). The exudates were recovered from the PVC container.

In order to localize flavonoids in different parts of the root, sterile *M. sativa* subsp. *sativa* cv. Sital plants were grown on Gibson medium as described in Bauer et al. (1994). Localization of the flavonoid-exuding region of the roots was performed according to Djordjevic et al. (1987) and Peters and Long (1988).

Source of flavonoids.

Authentic flavonoids were purchased from Extrasynthese SA (Genay, France). Other flavonoids used were isolated as described below.

Extract preparation.

Exudates and complete roots grounded in liquid nitrogen were stored at -80°C.

Root extract.

Approximately 800 g of the fresh samples ground in liquid nitrogen was exhaustively extracted with 70% aqueous etha-

nol by maceration to determine the flavonoid content in the roots. The solvent was evaporated under reduced pressure. The residue (approximately 30 g) was suspended in water and successively extracted with diethyl ether and *n*-butanol. After concentration of the extracts, 2.5 g of the ether extract, 8.5 g of the butanol extract, and 15.0 g of the aqueous residue were recovered.

Exudate extract.

The exudate was lyophilized, suspended in water, and, as for the root extracts, successively extracted with diethyl ether and *n*-butanol. The amount of extract recovered was 24.0 mg for the ether extract, 86.0 mg for the butanol extract, and 1.6 g for the aqueous residue.

Measurement of the *nod* gene inducing activity.

The capacity to induce *nod* gene expression of the extracts was monitored with *S. meliloti* JM57 (Mulligan and Long 1985) harboring the plasmid pKSK5 containing the *S. meliloti nodD1* gene, plasmid pGM108 carrying the *S. meliloti nodD2* gene, or plasmid pHB264 containing the *Rhizobium* sp. MPIK3030 *nodD1* gene (Györgypal et al. 1991), as specified. Bacteria were grown overnight to mid-log phase and diluted to an A₆₀₀ of 0.2 in 0.5 ml of YTB medium (Orosz et al. 1973) containing 5 µl of the extracts to be tested and the appropriate antibiotics if needed. All extracts were diluted at 2 mg/ml, except the aqueous extract, which was used at 6 mg/ml. After incubation for 16 h at 28°C, the cultures were monitored for β-galactosidase activity of the *nodC::lacZ* gene fusion as described (Miller 1972). All assays with the extracts were conducted at least three times (with 10 samples in each experiment) and statistical analysis of the data was performed with appropriate controls in each experiment. We used the solvent employed for the dilution of extracts, MeOH:H₂O (50:50 [vol/vol]) for negative control and the flavonoid DHF for positive control (data not shown).

Analytical and preparative HPLC.

Analytical HPLC was carried out as previously described (Coronado et al. 1995) for the root extracts and exudates. For the analysis of the different parts of the root, we used from 0 to 10 min a linear gradient solvent system from 40:60:0.1 (vol/vol/vol) MeOH:H₂O:TFA to 60:40:0.1 (vol/vol/vol) MeOH:H₂O:TFA. From 10 to 20 min, the analysis was continued isocratically at this concentration, and from 20 to 30 min a new linear gradient was applied to 100:0.1 (vol/vol) MeOH:TFA.

For the preparative HPLC we used a Waters system (Millipore, Milford, MA) equipped with a PrepPak 25 × 100 mm C18 6 µm column and radial compression at 700 to 800 lb/in², eluted at 5 ml · min⁻¹ from 0 to 40 min with a linear gradient solvent system from 40:60:0.1 (vol/vol/vol) methanol:water:trifluoroacetic acid to 80:20:0.1 (vol/vol/vol) methanol:water:trifluoroacetic acid (system A), or from 0 to 40 min with a linear gradient solvent system from 45:55:0.1 (vol/vol/vol) methanol:water:trifluoroacetic acid to 50:50:0.1 (vol/vol/vol) methanol:water:trifluoroacetic acid (system B). Eluting compounds were monitored with a Waters 600E system controller and a Waters 484 Tunable Absorbance Detector/UV, which measured absorbance at 254 or 340 nm, and every peak was separated.

Flavonoid identification in the root extracts.

Structural features of the purified flavonoids were examined by UV absorption spectroscopy in methanol (Markham and Mabry 1975) with a Lambda 5 UV/VIS Spectrophotometer (Perkin-Elmer, Norwalk, CT). The HPLC detection (Photodiode Array Analysis) was also used for the elucidation of the UV spectra of peaks detected in HPLC chromatogram, by comparing with those of standards. Co-injection with standards was also employed.

The mass spectra analysis was done according to the classic fragmentations type Retro Diels-Alder (Mabry and Markham 1975).

EIMS and NMR data were obtained as previously described (Coronado et al. 1995).

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