

Flavonoids from shoots and roots of *Trifolium repens* (white clover) grown in presence or absence of the arbuscular mycorrhizal fungus *Glomus intraradices*

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Abstract

White clover (*Trifolium repens*) plants were grown in the presence or absence of the arbuscular mycorrhizal fungus *Glomus intraradices*. Flavones, 4',5,6,7,8-pentahydroxy-3-methoxyflavone and 5,6,7,8-tetrahydroxy-3-methoxyflavone, as well as two flavones 3,7-dihydroxy-4'-methoxyflavone and 5,6,7,8-tetrahydroxy-4'-methoxyflavone never previously reported in plants, were isolated. The known 3,5,6,7,8-pentahydroxy-4'-methoxyflavone, 2',3',4',5',6'-pentahydroxy-chalcone, 6-hydroxykaempferol, 4',5,6,7,8-pentahydroxyflavone and 3,4'-dimethoxykaempferol were also obtained. Analysis of extracts obtained from roots and shoots revealed that the compositions of the flavonoid mixtures varied with growing conditions. Quercetin, acacetin and rhamnetin accumulated in roots of inoculated plants, whereas they were not detected in non-inoculated plants.

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Keywords: White clover; *Trifolium repens*; Leguminosae; Shoots; Roots; Flavonoids; Flavones; Chalcone; *Glomus intraradices*; 4',5,6,7,8-Pentahydroxy-3-methoxyflavone; 5,6,7,8-Tetrahydroxy-3-methoxyflavone; 3,7-Dihydroxy-4'-methoxyflavone; 5,6,7,8-Tetrahydroxy-4'-methoxyflavone

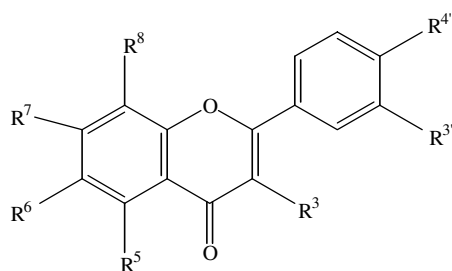
1. Introduction

As part of a project related to determining the role of flavonoids in symbiotic and parasitic relationships between plants and microbes (Fracchia et al., 2000, 2003, 2004), we isolated and characterized the flavonoids present in *Trifolium repens* (white clover). This plant was grown either in the presence or absence of the arbuscular mycorrhizal (AM) fungus *Glomus intraradices*. In this paper, we report the isolation from the shoots of non-inoculated *T. repens* of a new flavone, i.e., 4',5,6,7,8-pentahydroxy-3-methoxyflavone

(Fig. 1, **1a**), as well as two flavones from its roots that have never previously been isolated from plants, i.e., 3,7-dihydroxy-4'-methoxyflavone (Fig. 1, **1d**) and 5,6,7,8-tetrahydroxy-4'-methoxyflavone (Fig. 1, **1e**), along with the known flavones, 3,5,6,7,8-pentahydroxy-4'-methoxyflavone (Fig. 1, **1b**), 2',3',4',5',6'-pentahydroxy-chalcone (Fig. 2, **1c**) and 3,4',5,6,7-pentahydroxyflavone (6-hydroxykaempferol; Fig. 1, **1f**). We also report the isolation, from shoots of AM colonized *T. repens*, of one new flavone, 5,6,7,8-tetrahydroxy-3-methoxyflavone (Fig. 1, **2a**) and two known flavones 4',5,6,7,8-pentahydroxyflavone (**2b**) and 5,7-dihydroxy-3,4'-dimethoxyflavone (3,4'-dimethoxykaempferol, **2c**). From roots of AM colonized *T. repens*, the known flavonoids 3,3',4',5,7-pentahydroxyflavone (quercetin, **2d**), 5,7-dihydroxy-4'-methoxyflavone (acacetin, **2e**) and

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Comp.	R ³	R ⁵	R ⁶	R ⁷	R ⁸	R ^{3'}	R ^{4'}
1a	OCH ₃	OH	OH	OH	OH	H	OH
1b	OH	OH	OH	OH	OH	H	OCH ₃
1d	OH	H	H	OH	H	H	OCH ₃
1e	H	OH	OH	OH	OH	H	OCH ₃
1f	OH	OH	OH	OH	H	H	OH
2a	OCH ₃	OH	OH	OH	OH	H	H
2b	H	OH	OH	OH	OH	H	OH
2c	OCH ₃	OH	H	OH	H	H	OCH ₃
2d	OH	OH	H	OH	H	OH	OH
2e	H	OH	H	OH	H	H	OCH ₃
2f	OH	OH	H	OCH ₃	H	OH	OH

Fig. 1. Structures of flavonoids isolated.

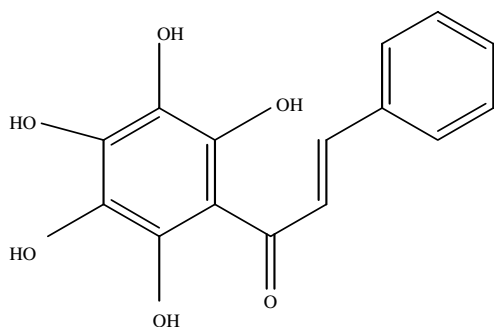


Fig. 2. Structure of compound 1c.

3,3',4',5-tetrahydroxy-7-methoxyflavone (rhamnetin, **2f**) together with flavone **1b** (Fig. 1) were found.

2. Results and discussion

Compound **1a** ($R_f = 0.70$) isolated from shoots of non-inoculated *T. repens* as a colourless solid, showed $[M]^+$ peaks at m/z 332 and at m/z 332.0530 in its EIMS and HREIMS, respectively, consistent with the molecular formula $C_{16}H_{12}O_8$ (m/z Calc.: 332.0532) which was further supported by analysis of its ^{13}C NMR spectrum (Table 1). The IR absorption bands at 3500 (broad), 1630, 1602 and 1492 cm^{-1} , and a positive ferric chloride test indicated that compound **1a** had free hydroxyl groups. The UV absorption maxima of **1a** in MeOH at 252 and 300 nm were typical of a flavonoid derivative (Mabry et al., 1970). Addition of NaOMe caused a shift

Table 1
 ^{13}C NMR spectral data (ppm) of **1a**, **1d**, **1e** and **2a**

C	1a	1d	1e	2a
2	152.6	145.2	163.0	150.8
3	143.3	139.0	105.1	141.5
4	176.5	173.1	180.8	177.3
4a	95.3	118.5	97.9	96.3
5	147.9	128.5	149.3	148.5
6	131.2	115.2	130.6	130.3
7	144.7	162.7	144.2	145.0
8	128.2	103.4	126.8	127.1
8a	150.8	156.6	152.7	152.1
1'	124.0	123.8	123.3	132.2
2'	126.6	128.4	128.1	128.5
3'	115.5	114.1	112.9	128.9
4'	160.3	163.1	163.7	130.8
5'	115.5	114.1	112.9	128.9
6'	126.6	128.4	128.1	128.5
3-OMe	60.5			59.9
4'-OMe		55.0	55.3	

in the UV absorption maxima of +68 nm in agreement with the presence of the HO group as a substituent at C-4' (Mabry et al., 1970). The 1H NMR spectrum of **1a** showed typical two *para*-coupled doublets with $J = 8.65$ Hz at δ 7.76 and 6.79, each integrating for two protons, which were assigned to the coupled H-2' and H-6', and H-3' and H-5', respectively. A sharp three-proton resonance for the one methoxyl group at δ 3.79 was also observed. The EIMS of **1a** had two retro-Diels–Alder fragments at m/z 184 (ring-A), 121 (HOC_6H_4CO , fragment B₂) and 148 ($CH_3OC=CC_6H_4OH$, ring-B) consistent with the presence of a methoxyl group at the C-3 position in the ring-A.

Compound **1d**, $R_f = 0.87$, was obtained from roots of non-inoculated *T. repens*. It gave a molecular ion $[M]^+$ at m/z 284 in the EI mass spectrum and at m/z 284.0683 in the HREIMS, corresponding to the molecular formula $C_{16}H_{12}O_5$ (Calc.: 284.0685). The EIMS of **1d** displayed two retro-Diels–Alder fragmentations, at m/z 135 [$CH_3OC_6H_4C=O$] and at m/z 136 [HOC_6H_3OCO], indicating the presence of a methoxyl group in ring-B. The absence of the signal at m/z 148 [$HOC=CC_6H_4OCH_3$] agrees with the presence of an hydroxyl group at C-3 in ring-A (i.e., EIMS of 3-hydroxyflavones in The Wiley Registry of Mass Spectral Data, 1996). The positive ferric chloride test indicated that compound **1d** had free hydroxyl groups. The structure of **1d** was thus deduced as shown.

Compound **1e** ($R_f = 0.62$) was isolated from roots of non-inoculated *T. repens* as a colourless solid. The IR absorption bands at 3300–3500 (broad), 1625, 1601 and 1490 cm^{-1} , and positive ferric chloride test indicated that compound **1e** had free hydroxyl groups. It gave $[M]^+$ peaks at m/z 316 and at m/z 316.0581 in its EIMS and HREIMS, respectively, consistent with the molecular formula $C_{16}H_{12}O_7$ (Calc.: 316.0583) further sup-

ported by the presence of 16 carbon signals in its ^{13}C NMR spectrum (Table 1). The ^1H NMR spectrum of **1e** showed signals for two *para*-coupled doublets with $J = 8.5$ Hz at δ 7.85 and 6.92, each integrating for two protons, which were assigned to the coupled H-2' and H-6', and H-3' and H-5', respectively. A sharp one-proton peak at δ 6.61, assigned to H-3, and another sharp three-proton singlet at δ 3.78 assigned to one methoxyl group C-4' were also observed. The EIMS of **1e** has two retro-Diels–Alder fragments at m/z 184 [$\text{M}^+ - \text{CH}_3\text{OPhC}=\text{CH}$], 135 [$\text{CH}_3\text{OPhC}=\text{O}$] and 132 [$\text{CH}_3\text{OPhC}=\text{CH}$] consistent, together with analysis of the ^1H NMR spectral data, with the presence of a methoxyl group at the C-4' position in the ring-B. Thus, from the foregoing spectral studies the structure of compound **1e** was established as 5,6,7,8-tetrahydroxy-4'-methoxyflavone (Fig. 1).

Compound **2a** ($R_f = 0.85$) was isolated from shoots of inoculated *T. repens* as a colourless solid. It gave a molecular ion [M^+] at m/z 316 in the EI mass spectrum and at m/z 316.0581 (Calc.: 316.0583) in HREIMS consistent with the molecular formula $\text{C}_{16}\text{H}_{12}\text{O}_7$, which was further supported by the presence of 16 carbon signals in its ^{13}C NMR spectrum (Table 1). The IR absorption bands at 3500 (broad), 1629, 1603 and 1491 cm^{-1} , and a positive ferric chloride test indicated that compound **2a** had free hydroxyl groups. The ^1H NMR spectrum of **2a** showed two typical *multiplet*-signals between δ 7.74–7.71 and 7.69–7.65 for two aromatic protons, the former corresponding to two aromatic protons, H-2' and H-6' and the latter, to three aromatic protons, H-3', H-4' and H-5'. A sharp three-proton singlet at δ 3.94 was also assigned to a methoxyl group at C-3'. The EIMS of **2a** displayed two retro-Diels–Alder fragments at m/z 184 [$\text{M}^+ - \text{PhC}=\text{COCH}_3$], 132 [$\text{PhC}=\text{COCH}_3$] and 105 [$\text{PhC}=\text{O}$], which together with analysis of the ^1H NMR spectroscopy data, indicated the presence of a methoxyl group at C-3 in ring-A; its deduced structure is shown in Fig. 1.

The structure of the known compounds **1b** (Ponce et al., 2004, submitted), **1c** (Ponce et al., 2004, submitted), **1f** (Horie et al., 1995; Hattori et al., 1992), **2b** (Wagner et al., 1965), **2c** (Zhu et al., 2000; Ma et al., 1999), **2d–2f** (Agrawal, 1989; The Wiley Registry of Mass Spectral Data, 1996) were established by comparison with literature data. Compounds **1d** and **1e** were never previously reported in plants and only the synthesis and partial characterization of **1d** had been previously reported (**1d**, Bellini and Venturella, 1958; Jerzmanowska and Michalska, 1961; **1e**, Miyahara et al., 1993) while compound **1e** was only mentioned in a QSAR study (Miyahara et al., 1993).

Most phytochemical studies on plants that are AM hosts have been performed with collected material from the field and they are colonized by wild AM fungal

population. Studies on the effect of AM symbiosis on plant physiology have been focussed on changes produced in roots but not in shoots. It is not clear from the literature if the AM fungus induces qualitative and/or quantitative changes in the composition of flavonoids in the plant.

Thus, this is the first comparative study of shoot and root flavonoid contents in white clover plants (*T. repens*) grown with and without a mycorrhizal fungus (*G. intraradices*). From shoots and roots of plants grown under the latter conditions, flavones never previously isolated from plants were obtained (**1a**, **1d**, and **1e**) together with **1b**, **1c** and **1f**. Compounds **1b** and **1c** were recently isolated from *Brassica alba* (Ponce et al., 2004, submitted). The roles of **1a–1e** in plant physiology and in plant–microbial interactions are still unknown. Compound **1f** was previously isolated from various plant materials (Hattori et al., 1992; Wollenweber et al., 1986, 1998). To the best of our knowledge no studies about the effect of **1f** on AM fungus have been reported.

From shoots and roots of plants grown with AM fungus, the flavonoids **2b–2f** isolated are known compounds. As far as we know, no studies about the effect of **2b**, **2c**, **2e** and **2f** on AM fungus have been reported. Quercetin (**2d**) has shown a positive effect on germination or hyphal length of different AM spores (Poulin et al., 1993), and together with acacetin (**2e**) and rhamnetin (**2f**) have been described as inhibitors of eukaryotic topoisomerase I (Boege et al., 1996). Acacetin also showed bacteriostatic activity (Fawe et al., 1998) and rhamnetin antifungal activity (Serra-Bonvehi et al., 1994). Compound **2b**, called ponkanetin, was previously isolated from various plant materials (Bellini et al., 1962; Chaliha et al., 1965) and its antineoplastic activity, cytotoxicity (Edwards et al., 1979) and mutagenicity (Nagao et al., 1981) have also been described. Compound **2c** was previously isolated from leaves and leaf exudates of different plants (Wollenweber and Doerr, 1995; Valant-Vetschera and Wollenweber, 1996; Wollenweber et al., 1997; Stevens et al., 1999) and its anti-poliovirus action (Robin et al., 2001), hepatoprotective and anti-*Helicobacter pylori* activity (Banskota et al., 2001) have been described.

As a conclusion, the different composition of the flavonoids extracted from shoots and roots of white clover (*T. repens*) grown with and without the AM fungus clearly shows that metabolism of these molecules is strongly affected when the plant is AM colonized.

The data presented suggest a clear link of the AM fungus with the regulation of flavonoid metabolism in shoots and roots of white clover colonized by *G. intraradices* with systemic effect on the plant. However, whether the changes in the flavonoid compositions either are a symptom or cause of colonization still has to be investigated.

3. Experimental

3.1. General

UV spectra (Shimadzu UV-1203) were recorded in MeOH, whereas IR spectra (Nicolet 510P FT-IR) were obtained as a KBr disk film. ^1H NMR (Bruker AM-500, 500 MHz) and ^{13}C NMR (Bruker AC-200, 75 MHz) spectra were acquired in DMSO- d_6 with TMS as internal standard, whereas EIMS (Shimadzu QP-5000/Gc-17A/DI-50) and HREIMS (VG-ZAB-VSEQ) were recorded at 70 eV (ionizing potential) using a direct inlet system. Samples of quercetin, crysin, and rutin were purchased from Aldrich. Luteolin, kaempferol and apigenin were purchased from Sigma. Morin was obtained from a natural source.

3.2. Plant material

Plants of *T. repens* were either grown in the presence or absence of *G. intraradices* (*repens* Mic^+ and *repens* Mic^- , respectively) in 100 ml pots of steam-sterilized soil mixed 2:1 (v:v) with vermiculite and inoculated with *G. intraradices* inoculum in the case of *repens* Mic^+ . Growth was carried out in a greenhouse with supplementary light provided by Sylvania incandescent and cold-white lamps, $400 \mu\text{E m}^{-2} \text{s}^{-1}$, 400–700 nm, with a 16/8 h day/light cycle at 25/19 °C and 50% relative humidity. Plants were harvested at a specific growth stage of 10 weeks, with shoots separated from roots. In each case, the plant materials were dried in oven at 75 °C for 72 h, prior to extraction.

3.3. Extraction and isolation

The dried plant materials were crushed and extracted exhaustively with EtOH at room temperature for 4 days (1.5 l of EtOH per each gram of dried material). The ethanolic extracts obtained from the shoots and roots of *T. repens* Mic^+ and *T. repens* Mic^- were filtered and evaporated to dryness (syrops, ≈ 3 g from roots and 15 g from shoots), then suspended in water (150 ml). Each aqueous suspension was then extracted successively with organic solvents (3×50 ml). The combined organic fractions obtained for each solvent: hexane (defatting), EtOAc and CH_2Cl_2 fractions, were evaporated to dryness and the residues individually analysed by TLC. TLC was carried out on precoated silica gel 60F₂₅₄ aluminium sheets (0.2 mm thickness, Merck) eluted with EtOAc– CH_2Cl_2 – HCO_2H (8:12:1). Chromatograms were visualized after drying (i) by UV light and (ii) by spraying with a solution containing 6 g vanillin and 3 ml H_2SO_4 in 197 ml of MeOH. The R_f values for TLC analysis of the authentic samples using the above solvent system were: rutin (0), morin (0.45), luteolin (0.63),

quercetin (0.67), crysin (0.88), apigenin (0.77), kaempferol (0.83) isorhamnetin (0.88).

The components of the EtOAc extracts (≈ 500 mg from shoots and 150 mg from roots) were separated by preparative layer chromatography on precoated silica gel 60F₂₅₄ aluminium sheets (0.2 mm thickness, Merck) (amount of sample applied < 25 mg; EtOAc– CH_2Cl_2 – HCO_2H 8:12:1 mixtures as eluent). After development the compounds were visualized as above using analytical TLC, then recovered by treating each portion of the adsorbent exhaustively with EtOAc, EtOAc–EtOH and EtOH (3×50 ml), respectively. After evaporation of the combined organic fractions, compounds (**1a**)–(**1f**), (**2a**)–(**2f**), were obtained (Figs. 1 and 2).

3.4. *T. repens* without mycorrhizae (Mic^-)

3.4.1. Shoots

3.4.1.1. 5,6,7,8-Tetrahydroxy-2-(4-hydroxyphenyl)-3-methoxy-4H-1-benzopyran-4-one or 4',5,6,7,8-pentahydroxy-3-methoxyflavone (**1a**). From dried shoots (46 g) of Mic^- , **1a** (7.10 mg) was isolated. $R_f = 0.70$; UV $\lambda_{\text{max}}^{\text{MeOH}}$ nm (log ϵ): 252 (4.32), 300 (4.04); +NaOMe: 252, 368. IR $\nu_{\text{max}}^{\text{film}}$ cm^{-1} : 3500 (broad), 1630, 1602, 1492. ^1H NMR (500 MHz, DMSO- d_6): δ 7.76 (2H, *d*, $J = 8.65$ Hz, H-2' and H-6'), 6.79 (2H, *d*, $J = 8.65$ Hz, H-3' and H-5'), 3.79 (3H, *s*, CH_3O); ^{13}C NMR (75 MHz, DMSO- d_6): see Table 1. EIMS 70 eV, m/z (%): 332 [$\text{M}]^+$ (100), 316 (9), 315 (7), 304 (15), 184 (16), 148 (18), 121 (29); HREIMS m/z 332.0530 (Calc. for $\text{C}_{16}\text{H}_{12}\text{O}_8$, 332.0532).

3.4.2. Roots

From dried roots (33 g) of Mic^- , **1b** (2.3 mg), **1c** (2.0 mg), **1d** (2.8 mg), **1e** (4.30 mg) and **1f** (2.5 mg) were isolated. Compounds **1b** and **1c** (Ponce et al., 2004, submitted) and **1f** (Horie et al., 1995; Hattori et al., 1992) were identified by comparison with the literature data.

3.4.2.1. 3,7-Dihydroxy-2-(4-methoxyphenyl)-4H-1-benzopyran-4-one or 3,7-dihydroxy-4'-methoxyflavone (**1d**). $R_f = 0.87$ (Bellini and Venturella, 1958; Jerzmanowska and Michalska, 1961); for ^{13}C NMR analysis (75 MHz, DMSO- d_6), see Table 1. EIMS 70 eV, m/z (%): 284 [$\text{M}]^+$ (100), 253 (25), 250 (17), 240 (12), 136 (26), 135 (25), 120 (7); HREIMS m/z 284.0683 (Calc. for $\text{C}_{16}\text{H}_{12}\text{O}_5$, 284.0685).

3.4.2.2. 5,6,7,8-Tetrahydroxy-2-(4-methoxyphenyl)-4H-1-benzopyran-4-one or 5,6,7,8-tetrahydroxy-4'-methoxyflavone (**1e**). $R_f = 0.62$ (Miyahara et al., 1993); UV $\lambda_{\text{max}}^{\text{MeOH}}$ nm (log ϵ): 250 (4.45), 302 (4.10). IR $\nu_{\text{max}}^{\text{film}}$ cm^{-1} : 3500 (broad), 1625, 1601, 1490. ^1H NMR (500 MHz, DMSO- d_6): δ 7.85 (2H, *d*, $J = 8.5$ Hz, H-2' and H-6'), 6.92 (2H, *d*, $J = 8.5$ Hz, H-3' and H-5'), 6.61 (1H, *s*, H-

3), 3.78 (3H, *s*, CH₃O); For ¹³C NMR (75 MHz, DMSO-*d*₆) analysis see Table 1. EIMS 70 eV, *m/z* (%): 316 [M]⁺ (100), 301 (8), 300 (5), 288 (8), 274 (10), 184 (12), 135 (12), 132 (20); HREIMS *m/z* 316.0581 (Calc. for C₁₆H₁₂O₇, 316.0583).

3.5. *T. repens* colonized by intraradices (*Mic*⁺)

3.5.1. Shoots

From dried shoots (54 g) of *Mic*⁺, **2a** (20.7 mg), **2b** (2.4 mg) and **2c** (4.2 mg) were isolated. Compounds **2b** (Wagner et al., 1965) and **2c** (Zhu et al., 2000; Ma et al., 1999) were identified by comparison of their physical properties with the literature values.

3.5.1.1. 5,6,7,8-Tetrahydroxy-3-methoxy-2-phenyl-4H-1-benzopyran-4-one *1* or 5,6,7,8-tetrahydroxy-3-methoxy-flavone (**2a**). *R*_f = 0.85; UV *v*_{max}^{MeOH} nm (log *ε*): 252 (4.53), 300 (4.21). IR *v*_{max}^{film} cm⁻¹: 3500 (broad), 1629, 1603, 1491. ¹H NMR (500 MHz, DMSO-*d*₆): δ 7.74–7.71 (2H, *m*, H-2' and H-6'), 7.69–7.65 (3H, *m*, H-3', H-4' and H-5'), 3.94 (3H, *s*, CH₃O); For ¹³C NMR (75 MHz, DMSO-*d*₆) analysis see Table 1. EIMS 70 eV, *m/z* (%): 316 [M]⁺ (100), 301 (6), 299 (5), 288 (9), 184 (18), 132 (23), 105 (24). HREIMS *m/z* 316.0581 (Calc. for C₁₆H₁₂O₇, 316.0583).

3.5.2. Roots

From dried roots (24 g) of *Mic*⁺, **1b** (4.0 mg), **2d** (5.0 mg), **2e** (6.0 mg) and **2f** (4.30 mg), were isolated. Compounds (**1b**) (Ponce et al., 2004, submitted), *quercetin* (**2d**), *acacetin* (**2e**) and *rhamnetin* (**2f**) also were identified by comparison of their physical properties with the literature values (The Wiley Registry of Mass Spectral Data, 1996; Agrawal, 1989; Mabry et al., 1970).

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