

Flavonoids from *Vicia faba* Seed Exudates

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From the seed exudates of *Vicia faba* L. (cv. Muchamiel) the flavonoid aglycones 7,3',4'-trihydroxyflavone, 7,4'-dihydroxyflavone, quercetin and kaempferol, and the flavonoid glycosides quercetin 7-glucoside and kaempferol 7-glucoside were identified. This is the first time that the flavonoids present in *Vicia* seed exudates are described. The study of the flavonoids present in legume seed and root exudates is especially important since these substances may act as chemical signals activating or inhibiting *Rhizobium* nodulation genes. In fact, the activating effect on *Rhizobium* nod genes of 7,3',4'-trihydroxyflavone and 7,3'-dihydroxyflavone has previously been reported. It is remarkable, that these compounds increase dramatically in mature pods, and these tissues might have an additional ecological role in the signal function on *Rhizobium* to establish the symbiosis.

Introduction

The first signal to establish the symbiosis, in the Leguminosae, is the induction of nodulation genes of *Rhizobium* by exudate compounds from the seed or root of the plant [1]. In the pairs of symbionts studied so far, the inducing compounds have been identified as plant flavonoids, but the specific flavonoid is different from each symbiotic pair. Thus, the following inducing compounds were isolated from plant exudates and identified as luteolin for alfalfa/*R. meliloti* [1], 7,4'-dihydroxyflavone for clover/*R. trifolii* [2] and daidzein and genistein from soybean/*Bradyrhizobium japonicum* [3]. By trying various flavonoids, eriodictyol and hesperetin were found to best induce on pea and *Vicia sativa* [4]. On the other hand, in the exudates there are compounds which inhibit the expression of the nodulation genes, so those of *R. meliloti* are inhibited by the flavonols quercetin and kaempferol [5]. One could think that the nodulation genes of *Rhizobium* are subjected to activation

and/or inhibition by different specific molecules of the host [6].

The aim of the present work is the identification of flavonoid compounds present in broad bean seed exudates.

Materials and Methods

Vicia faba (cv. Muchamiel) seeds (10 kg) were rinsed in water for imbibition. After 24 h, water was separated, filtered and extracted with Et₂O. This extract was chromatographed on Sephadex LH-20, and two flavonoid fractions were obtained. Six compounds (**1–6**) were then isolated by paper chromatography on Whatman No. 1 with 15% HOAc and 30% HOAc. The different compounds were identified by their UV data, chromatographic comparisons with authentic markers (HPLC and TLC), and in the case of glucosides by enzymic hydrolysis with β-D-glucosidase. HPLC analysis: the Et₂O extracts were concentrated under reduced pressure and redissolved in MeOH. These MeOH extracts and the isolated flavonoid aglycones were analyzed on a reversed-phase column LiChrospher 100 RP-18 (5 μm) using as mobile phase MeOH–H₂O (1:1) (H₂O with 5% HCOOH) isocratically with a flow rate of 1 ml/min and detection with a photodiode array detector. TLC analysis: flavonoid aglycones and glycosides were TLC analyzed on cellulose plates with 30% HOAc. The different spots were visualized under UV light (360 nm). Enzymic hydrolysis: compounds **5** and **6** were dissolved in 1 M acetate buffer pH 4.5 and β-D-glucosidase (Sigma) was added and incubated at 30 °C for 6 h [8]. This was then extracted with Et₂O and flavonoid aglycones were analyzed by HPLC and TLC.

Results and Discussion

From broad bean seed exudates two blue fluorescent and four yellow fluorescent flavonoids were isolated. The blue compounds **1–2** under UV light (360 nm) changed to bright yellow when fuming with ammonia. Their UV spectra in methanol, and after addition of the classical shift reagents [7] indicated that these compounds had no free hydroxyl in 5-position, as well as the presence of free hydroxyls in 7- and 4'-positions, and the existence of an additional hydroxyl at 3'- in compound **1**. UV data: compound **1**: UV λ_{max} in MeOH (nm) 340, 308,

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257sh, 237sh; +NaOMe: 402, 342sh, 311sh, 257; +AlCl₃: 452sh, 364, 308, 239; +AlCl₃ + HCl: 412sh, 357, 307, 238; +NaOAc: 376, 310, 255; +NaOAc + H₃BO₃: 363, 305, 258sh. Compound **2**: UV λ_{\max} MeOH (nm) 329, 312sh, 254sh, 230sh; +NaOMe: 386, 329, 264sh, 251; +AlCl₃: 382sh, 328, 312sh, 254sh, 234; +AlCl₃ + HCl: 398sh, 328sh, 313, 255sh, 237; +NaOAc: 373, 320sh, 308, 263; +NaOAc + H₃BO₃: 329, 313sh, 256sh. These substances were identified as 7,3',4'-trihydroxyflavone (**1**) and 7,4'-dihydroxyflavone (**2**) by chromatographic comparisons (TLC and HPLC) with authentic markers (Roth).

In addition, four yellow fluorescent compounds were isolated. Their chromatographic behaviour on 2D PC [7, 8] suggested that they were two flavonol aglycones (**3–4**) and two flavonol 7-glycosides (**5–6**). The aglycones were identified as quercetin and kaempferol by their UV spectra and chromatographic comparisons with authentic markers, and the glycosides as quercetin and kaempferol 7- β -D-glucosides, by their UV spectra and enzymic hydrolysis with β -D-glucosidase which yielded the corresponding aglycones. UV data: Compound **3**: UV λ_{\max} in MeOH (nm) 371, 302sh, 267sh, 254; +NaOMe: 321 (decomposition), 247sh; +AlCl₃: 458, 334, 300sh, 271; +AlCl₃ + HCl: 429, 358, 300sh, 266; +NaOAc: 395 (decomposition), 329, 275, 256sh; +NaOAc + H₃BO₃: 388, 302sh, 260. Compound **4**: UV λ_{\max} MeOH (nm) 368, 322sh, 295sh, 267, 253sh; +NaOMe: 420 (decomposition), 316, 279; +AlCl₃: 425, 350, 303sh, 269, 258sh; +AlCl₃ + HCl: 423, 348, 300sh, 269, 255sh; +NaOAc: 386, 303, 275; +NaOAc + H₃BO₃: 372, 319sh, 297sh, 268. Compound **5**: UV λ_{\max} in MeOH (nm) 371, 268sh, 256; +NaOMe: 450 (decomposition), 365, 290, 243sh; +AlCl₃: 455, 339, 274, 259sh; +AlCl₃ + HCl: 426, 364, 303sh, 268; +NaOAc: 425sh (decomposition), 379, 285; +NaOAc + H₃BO₃: 385, 289sh, 260. Compound **6**: UV λ_{\max} in MeOH (nm) 364, 323, 265, 252; +NaOMe: 425 (decomposition), 335sh, 266, 245; +AlCl₃: 420, 352, 297sh, 265, 260sh; +AlCl₃ + HCl: 422, 349, 299sh, 266, 257sh, 240sh; +NaOAc: 417sh, 375, 320, 260; +NaOAc + H₃BO₃: 370, 324sh, 265sh. The concentration of these yellow flavonoids in the seed exudates increased threefold after 48 h of seed imbibition while the blue fluorescent flavonoids decreased. This decrease support previous results [9].

The occurrence of quercetin and kaempferol (and related unidentified glycosides) in the skin of broad bean seeds has previously been depicted [10–11]. By contrast, 7,3',4'-trihydroxyflavone and 7,4'-dihydroxyflavone have not been hitherto detected in seed tissues, although the occurrence of these substances in legume leaves has been reported [12].

Compound **2** has previously been described as the nodulation inducing substance from *Trifolium repens* [2] while a commercial sample of compound **1** activated *Rhizobium leguminosarum* nod genes [6] but this later compound was not detected in any seed or root exudate so far. Thus the presence of these substances in *Vicia faba* seed exudates might be in association with the establishment of the symbiotic process in broad bean. Recently, the presence of these blue fluorescent substances in *Vicia faba* pods, in which seeds had been removed, has been demonstrated (Tomás-Barberán *et al.*, unpublished results). These substances are present in the green pods at low concentrations (1–10 $\mu\text{g}/100$ g fresh plant material). However, they increase dramatically during the senescence period (more than 300 $\mu\text{g}/100$ g fresh plant material), when the pods become redish and then dark-brown (Fig. 1). A physiological explanation to this increase, would be related to the effect of these aglycones as inducers of nodulation genes in *Rhizobium*, since mature pods can fall down to the soil and flavo-

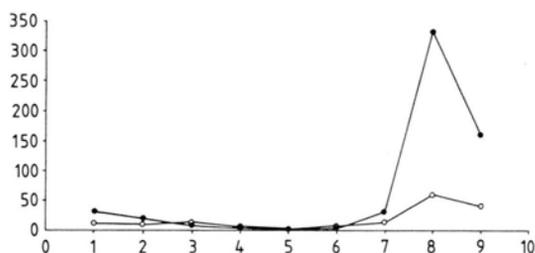


Fig. 1. Content (μg of flavonoid/100 g fresh pod without seeds) of 7,3',4'-trihydroxyflavone (●) and 7,4'-dihydroxyflavone (○) in *Vicia faba* pods. Variation with maturity. Numbers 1–9 represent groups of pods with different maturity. (1) Pods 1–6 cm long; (2) 6–8 cm; (3) 8–10 cm; (4) 10–13 cm; (5) 13–15 cm; (6) green pods around 20 cm with fully developed seeds; (7) fully developed pods with redish spots; (8) fully developed pods with intense red colour; (9) dark-brown pods.

noids may exert their function on *Rhizobium* species. This would be supported by the fact that these compounds are active at very small concentrations (10⁻¹⁰ M) [6].

The fact that these exudates contain inducing flavones (**1–2**) and antagonistic flavonoids as quercetin and kaempferol and their 7-glucosides (**3–6**) suggest that the genetic regulation of *Rhizo-*

bium is controlled by different specific molecules which co-occur in the host.

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