

Original Research

Alfalfa (*Medicago sativa* L.) Apigenin Glycosides and Their Effect on the Pea Aphid (*Acyrtosiphon pisum*)

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Abstract

Flavonoids are a group of secondary metabolites found in most families. They are known to have important physiological functions in plants by protecting them against biotic stresses. Liquid chromatography (HPLC) was used to determine the flavonoid profiles, especially apigenin glycosides, their total concentration, as well as changes in the amount of six flavones found in the aerial parts of alfalfa (*Medicago sativa* L.) (Fabaceae) Radius cv. for three vegetative stages, uninfested and infested by the pea aphid (*Acyrtosiphon pisum* Harris) (Homoptera: Aphididae). It has been shown that both control and infested green aerial parts of alfalfa plants had similar flavonoid profiles. The dominant flavonoid of alfalfa was compound 7-*O*-[2-*O*-feruloyl- β -D-glucuronopyranosyl(1 \rightarrow 2)-*O*- β -D-glucuronopyranosyl]-4'-*O*- β -D-glucuronopyranosideapigenin. Compound 4'-*O*- β -D-glucuronopyranosideapigenin was present in the smallest amounts. The total concentration of flavones was rather high and ranged from 10.32 to 12.28 mg/g d.m., but there were no significant differences between uninfested and infested alfalfa plants. There was a negative correlation between the concentration of total apigenin glycosides in the alfalfa plants and pea aphid abundance and phloem sap ingestion. This finding may indicate the importance of apigenin glycoside forms as nutritional compounds.

Keywords: *Medicago sativa*, flavonoids, apigenin glycosides, herbivore, *Acyrtosiphon pisum*

Introduction

Alfalfa (*Medicago sativa* L. (Fabaceae)) is an important crop used as feed for livestock [1]. One of the most serious pests of alfalfa is the pea aphid (*Acyrtosiphon pisum* Harris), and previous research demonstrated a reduction in alfalfa yields as a function of the pea aphid population level [2]. Moreover, the pea aphid is an important vector of legume viral diseases [3].

In addition to the nutritional components (proteins and carbohydrates) that are important in the use of alfalfa and other plants as animal feed or food supplements, the plants produce a variety of secondary metabolites. Many of these secondary metabolites help to protect the plant against herbivores [4-6], and it has been shown that plant metabolites can also influence the choice of food sources by insect herbivores [7-9].

Alfalfa contains numerous secondary metabolites [10-13], but they are not yet fully characterized. The most studied secondary metabolite groups in alfalfa are the carotenoids

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and saponins [14-16]. Another important group of secondary metabolites in alfalfa are phenolic compounds [17]. The toxicity of phenolic compounds to herbivores and plant pathogens has been the focus of substantial research [18-21].

Phenolic compounds in plants include the flavonoids. Flavonoids are usually found in plants as glycosides, i.e. provided with sugar substituents such as galactose, rhamnose or glucose, or glycoside malonates [22], and they are found in an almost bewildering diversity of forms [23]. Recent work on alfalfa flavonoids revealed that they consist of among other things, apigenin glycosides. Apigenin glycosides have been previously separated from alfalfa aerial parts and their structures were confirmed by UV, MS, and NMR spectroscopy [12, 13]. These compounds possessed glucuronic acid in the sugar chain. Some of them were acylated with ferulic or coumaric acids (Fig. 1). Apigenin glycosides play a very important role in plant development and physiology, especially during their interactions with other living organisms. Flavonoid glycosides and free aglycones are involved in pathogenic and symbiotic interactions with microorganisms [24, 25]. They also affect plant-plant and plant-insect interactions [26]. Flavonoids are also thought to affect insect behaviour and performance [27-29], and many of them are being tested for their ability to repel or deter insects [27, 30-33]. Flavonoids can also modulate the feeding behaviour of insects [34-36]. However, the effect of increasing the levels of specific flavonoids in plants on insects is unknown.

Although a number of flavonoids from different parts of the alfalfa plant have been identified [12, 13, 37], our understanding about the specific flavonoids present in alfalfa, their concentrations and effects on insects remains poor. The present study, therefore, analyzed the total flavonoid content and apigenin glycosides from the green aerial parts

of alfalfa and investigated the influence of these compounds on the pea aphid.

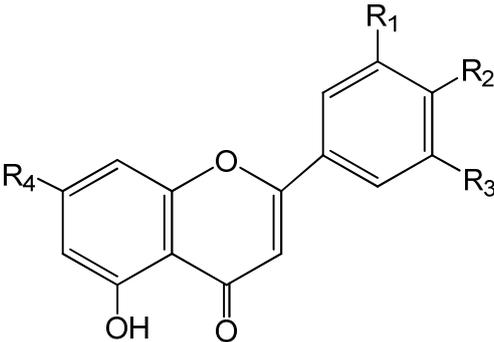
Materials and Methods

Plant Material

Alfalfa cv. Radius (*Medicago sativa* L. ssp. falcata x ssp. sativa), which has a high saponin content (65% of dry matter), was used in this study. Seed samples were obtained from the Plant Breeding and Acclimatization Institute (IHAR) in Radzików/Błonie (near Warsaw, Poland). Seeds were germinated in an environmental chamber at $21\pm 1^\circ\text{C}$, with 16 h daylight and 8 h of darkness, and 70% relative humidity. Plants were grown in $7\times 7\times 9$ cm plastic pots (one plant per pot) filled with fine garden soil, commonly used for greenhouse experiments. The plants were regularly watered, and no extra fertilizer was added. The aerial parts of plants (in three vegetative stages: 6-, 9-, 12-month-old) that were uninfested or infested by *Acyrtosiphon pisum* were used in the experiments.

Aphids

The pea aphids came from a stock culture kept at the University of Podlasie in Siedlce, Poland. The aphids were collected from a laboratory culture reared on broad bean seedlings [*Vicia faba* L. var. Start (Fabaceae)] in an environmental chamber at $21\pm 1^\circ\text{C}$, with 16 h daylight and 8 h of darkness, and 70% relative humidity. Before the experiments, female *A. pisum* were maintained on alfalfa cv. Radius for one full generation. The adult apterous females were then used in the experiments [38].



Comp.	R ₁	R ₂	R ₃	R ₄
1	-H	-OGluA	-H	-OH
2	-H	-OGluA	-H	-OGluA(2→1)GluA-2-O-Feruloyl
3	-H	-OGluA(2→1)GluA-2-O-Feruloyl	-H	-OGluA
4	-H	-OGluA(2→1)GluA-2-O-p-Coumaroyl	-H	-OGluA
5	-H	-OH	-H	-OGluA
6	-H	-OGluA(2→1)GluA-2-O-Feruloyl	-H	-OH

Fig. 1. Chemical formula of analyzed alfalfa flavones.

Entomological Observations

The entomological observations were carried out using an environmental chamber at $21 \pm 1^\circ\text{C}$, 16 h daylight and 8 h of darkness, and 70% relative humidity. Plexiglass cages $10 \times 10 \times 30$ cm with a cheesecloth cover were used. The adult apterous females were caged (one female per cage, one cage per plant) on the abaxial side of the youngest, fully expanded leaves of the alfalfa, and allowed to deposit nymphs. After 24 h, all but one nymph was removed from each plant. Each plant age group (6-, 9-, and 12-month-old) was represented by 10 independent replicate plants. Aphids on each plant were counted after 15 days.

Electrical Penetration Graphs

Feeding behaviour of the pea aphid on the *Radius cv.* was monitored using the Electrical Penetration Graphs (EPG) technique according to Tjallingii [39, 40]. The experiments were run for 8 h for 10 aphids, on 10 different plants placed in a Faraday cage. Apterous adult aphids were connected to a DC EPG amplifier (type Giga 4) by 2 cm gold wire, 20 μm in diameter, and approximately 2-3 cm long, and attached to the aphid with silver conductive paint (Demetron, L2027, Darmstadt, Germany). Another electrode was introduced into the soil. The studied insects were starved in a Petri dish for two hours before the recordings and then were placed on the abaxial surface of the youngest, fully expanded leaves of the plants. Aphid feeding activity was recorded using the data acquisition option on a PC and analyzed using STYLET 2.2 software (Agricultural University, Wageningen, The Netherlands). The duration and number of the following behavioural aphid activities were determined: non-probing (Np pattern; aphids did not start probing), probing (intercellular stylet penetration activities; path C pattern – pathway; penetration of peripheral tissues – epidermis and mesophyll), sieve element penetration (E1 pattern), ingestion of phloem sap (E2 pattern – aphid feeding), and xylem sap ingestion (G pattern).

High-Performance Liquid Chromatography of Flavonoids

Aerial parts of the 6-, 9-, and 12-month-old plants that were noninfested or infested by *A. pisum* were harvested, freeze-dried, ground, and kept in a desiccator in darkness until analyzed. Flavonoid analyses, including total flavonoid content and apigenin glycosides, followed Oleszek and Stochmal [41]. In short, each extract was obtained using the ASE 200 Accelerated Solvent Extractor (Dionex Corporation, Sunnyvale, USA) for 20 minutes with 70% methanol. The extracts were concentrated at 40°C on a rotary evaporator until the methanol was removed and then loaded on C_{18} cartridges (Waters, Poland) preconditioned with water. The flavonoids were then successively washed from the cartridges with water and 40% methanol. Methanolic fractions were evaporated on a rotary evapora-

tor at 40°C until dry, and the residue was redissolved in 1 ml of 40% MeOH. Extracts were analyzed using high-performance liquid chromatography (HPLC) according to Oleszek and Stochmal [41]. Flavonoids were separated using a Waters HPLC system, consisting of a model 616 pump and 99 G PAD detector (Waters Corporation, Milford, USA). The Millennium Chromatography Manager software (Waters Corporation) was used to monitor chromatographic parameters and to process the data. The alfalfa samples were applied to a Eurospher PD 82 column and eluted at 1 ml min^{-1} with a linear gradient of 1% phosphoric acid in water: 40% acetonitrile in 1% H_3PO_4 (65:35%), increasing to 0:100% over 60 min. Chromatograms were registered and integrated at 350 nm. Standards of apigenin and apigenin glycoside, purchased from the Biochemical Laboratory Institute of Soil Science and Plant Cultivation (Puławy, Poland), were used for calibration curve preparation. The total flavonoid concentration was calculated from the total integration area (350 nm) using the calibration curve of apigenin glycoside.

Statistical Analysis

The differences in foliar chemistry between uninfested (control) and infested alfalfa plants were analyzed with Student's *t* test. The differences in levels of the apigenin glycosides in the studied alfalfa plants were subjected to one-way ANOVA followed by the post-hoc Duncan test. Influence of the apigenin glycosides on pea aphid abundance and feeding activities were analyzed using Spearman rank correlation. The Statistica program for Windows v. 6.0 [42] was used for all statistical analyses.

Results

Variation in Flavonoid Profiles and Content among the Studied Alfalfa Plants

Pea aphid uninfested and infested 6-, 9-, and 12-month-old alfalfa plants had similar flavonoid profiles. Apigenin glycosides were identified on the basis of the absorption spectra of the chromatograms. Previous HPLC-MS studies have been conducted with these compounds. Two compounds (1 and 5) were non-acylated and the rest were acylated. Compounds 2, 3 and 6 were acylated with ferulic acid, compound 4 with coumaric acid. Flavones, including:

- (1) 4'-*O*- β -D-glucuronopyranosideapigenin;
- (2) 7-*O*-[2-*O*-feruloyl- β -D-glucuronopyranosyl(1 \rightarrow 2)-*O*- β -D-glucuronopyranosyl]-4'-*O*- β -D-glucuronopyranosideapigenin;
- (3) 7-*O*- β -D-glucuronopyranosyl-4'-*O*-[2'-*O*-feruloyl-*O*- β -D-glucuronopyranosyl(1 \rightarrow 2)-*O*- β -D-glucuronopyranoside]apigenin;
- (4) 7-*O*- β -D-glucuronopyranosyl-4'-*O*-[2'-*O*-*p*-coumaroyl-*O*- β -D-glucuronopyranosyl(1 \rightarrow 2)-*O*- β -D-glucuronopyranoside]apigenin;
- (5) 7-*O*- β -D-glucuronopyranosideapigenin; and

Table 1. Foliar chemistry (mg/g dry matter \pm SD)(average for Radius cv. in three studied stages).

Substance category	Alfalfa plants		Statistics	
	Unifested	Infested	t	P-value
Total flavonoids	10.32 \pm 1.72	12.28 \pm 0.58	1.868	0.135
Total apigenin glycosides	3.55 \pm 0.52	3.99 \pm 0.10	1.435	0.224
Apigenin nonacylated	0.77 \pm 0.05	0.85 \pm 0.06	1.408	0.232
Apigenin acylated	2.78 \pm 0.48	3.14 \pm 0.14	1.263	0.275

Table 2. The concentration (mg/g dry matter \pm SD) of individual apigenin glycosides (average for Radius cv. in three studied stages).

Apigenin glycosides*	Alfalfa plants		Statistics	
	Unifested	Infested	t	P-value
(1)	0.131 \pm 0.077	0.190 \pm 0.030	1.235	0.284
(2)	1.519 \pm 0.319	1.699 \pm 0.168	0.865	0.435
(3)	0.444 \pm 0.054	0.409 \pm 0.073	0.666	0.541
(4)	0.189 \pm 0.046	0.242 \pm 0.039	1.546	0.197
(5)	0.640 \pm 0.028	0.655 \pm 0.080	0.293	0.784
(6)	0.624 \pm 0.089	0.791 \pm 0.131	1.832	0.141

*(1) 4'-O- β -D-glucuronopyranosideapigenin;

(2) 7-O-[2-O-feruloyl- β -D-glucuronopyranosyl(1 \rightarrow 2)-O- β -D-glucuronopyranosyl]-4'-O- β -D-glucuronopyranosideapigenin;

(3) 7-O- β -D-glucuronopyranosyl-4'-O-[2'-O-feruloyl-O- β -D-glucuronopyranosyl(1 \rightarrow 2)-O- β -D-glucuronopyranoside]apigenin;

(4) 7-O- β -D-glucuronopyranosyl-4'-O-[2'-O-p-coumaroyl-O- β -D-glucuronopyranosyl(1 \rightarrow 2)-O- β -D-glucuronopyranoside]apigenin;

(5) 7-O- β -D-glucuronopyranosideapigenin;

(6) 4'-O-[2'-O-feruloyl- β -D-glucuronopyranosyl(1 \rightarrow 2)]-O- β -D-glucuronopyranoside]apigenin.

(6) 4'-O-[2'-O-feruloyl- β -D-glucuronopyranosyl(1 \rightarrow 2)]-O- β -D-glucuronopyranoside]apigenin were identified in alfalfa cv. Radius.

The total concentration of flavonoids was high but did not differ significantly between aphid-infested and uninfested alfalfa plants (Table 1). The total concentration of apigenin glycosides, non-acylated apigenin and acylated apigenin also did not differ significantly between aphid-infested and uninfested alfalfa plants (Table 1).

Flavonoid analyses revealed substantial individual variation (Table 2). It was shown that (2) compound was the dominant apigenin glycoside of pea aphid infested and uninfested alfalfa plants. Compound (1) was present in the smallest amounts (Table 2).

Abundance and Feeding Behaviour of the Pea Aphid on the Studied Alfalfa Plants

There were clear differences in abundance of the pea aphid on all the studied alfalfa plants. The highest number of pea aphids was found on 12-month-old plants and the lowest aphid performance was on the 6-month-old plants (ANOVA, $P < 0.05$) (Fig. 2). Pea aphid abundance on alfalfa cv. Radius was correlated with the concentration of total apigenin glycosides in the alfalfa plants (Spearman rank

correlation $r_s = -0.903$, $p < 0.05$), but there were no correlations between pea aphid abundance and the concentration of total flavonoid compounds (Spearman rank correlation $r_s = 0.23$, $p = 0.658$), acylated flavones (Spearman rank correlation $r_s = 0.41$, $p = 0.088$) and non-acylated flavones (Spearman rank correlation $r_s = 0.43$, $p = 0.078$).

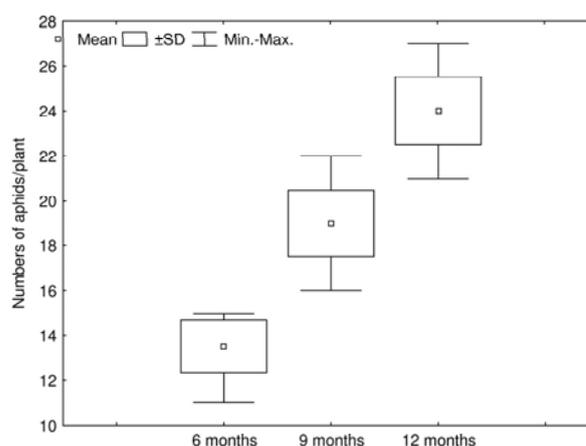


Fig. 2. Abundance of the pea aphid on alfalfa plants of three different ages.

Pea aphid feeding behaviour on alfalfa cv. Radius was affected by apigenin glycosides. A negative correlation was found between pea aphid phloem sap ingestion and the concentration of apigenin glycosides ($r_s = -0.681$, $P < 0.05$) in alfalfa plants.

Discussion

To date, relatively little is known about alfalfa flavonoids with respect to their chemistry and biological activity. Not much has been known about the concentration of flavones in alfalfa, especially apigenin glycosides and their role in insect nutrition. Their effect on the *A. pisum* aphid has not been extensively studied. In the present study of the aerial parts of alfalfa cv. Radius, six flavonoids (as apigenin glycoside) were identified. Apigenin glycosides are rare in plants, especially those with apigenin as aglycone. Some of them have been identified in only a few plant sources, among others in *Medicago radiata* [43]. The apigenin glycosides we analyzed were previously reported and identified in alfalfa var Artal and/or Boja, and on the basis of their spectral data, their structures were established [12, 13, 44].

In our study, the flavonoid profiles were similar to those reported by Stochmal and Oleszek [45]. The total flavonoid concentration we reported in alfalfa's aerial parts was rather high compared to other plant sources [46]. The concentration of individual apigenin glycosides we reported differed from what Stochmal and Oleszek's [45] study found in Radius. This is most likely associated with the different conditions of the experiments.

In our study, we reported that the total concentration of flavones in pea aphid infested and uninfested alfalfa plants were not significantly different. Similar trends were shown for alfalfa cv. Julius plants uninfested and infested by *Spodoptera littoralis* [28], but Kain and Biggs [47] observed differences in the concentration of the isoflavone coumestrol in alfalfa herbage uninfested and infested by the pea aphid and the bluegreen alfalfa aphid (*Acyrtosiphon kondoi*).

The content of various xenobiotics in plant tissues affect aphids' behaviour and performance [21, 48-51]. It has been demonstrated that plant flavonoids affect performance, fecundity and survival of herbivores [52-55]. The high mortality of insects was caused by apigenin [56]. In our study, we noted that apigenin glycosides modify the behaviour of the pea aphid. There was a negative correlation between the concentration of total apigenin glycosides in the alfalfa plants and pea aphid abundance. We showed that all apigenin glycosides have antifeedant and growth inhibitory effects on the pea aphid. Earlier investigations have shown that apigenin and apigenin glycosides are feeding deterrents to generalist herbivores [57, 58]. It has been demonstrated that apigenin and its glucoside derivatives provide resistance to feeding by insects [28, 59, 60].

Alfalfa flavones are a mixture of acylated and non-acylated forms. In our study, we observed that both types of these forms grew in the same concentrations in infested

alfalfa plants as in uninfested ones. Additionally, the concentration of acylated forms was much higher than non-acylated ones. This may suggest that acylated apigenin glycosides are more important for the plant than non-acylated forms in protecting against herbivores.

The chemicals identified in this study appear to be biologically active and could be used to alter the behaviour of pest insects. These research findings suggest further work is needed to reveal the complexity of biological activity exerted by the mixture of flavonoids.

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