Introduction

Alfalfa (*Medicago sativa* L. (Fabaceae)) is an important agricultural and commercial crop used as feed for livestock [1]. The pea aphid *Acyrthosiphon pisum* Harris (Hemiptera: Aphididae) is one of the most important pests of alfalfa. Moreover, it is an important vector of plant virus diseases [2]. Previous research demonstrated a reduction in alfalfa yields as a function of the pea aphid population level [3].

In addition to the nutritional components like proteins or carbohydrates, alfalfa contains a variety of secondary metabolites [4-7] that show biological activities [8-10]. But they are not yet fully characterized, and our understanding of alfalfa – *A. pisum* interactions.
about their concentration and effect on insects remains poor. In alfalfa a potentially interesting class of natural molecules are the flavonoids, a group of secondary plant metabolites that in plant cells are present as glycosides [11]. Recent work on alfalfa flavonoids has revealed that they consist of apigenin, luteolin, tricin, and chrysoeriol glycosides and possess glucuronic acid in the sugar chain. Apigenin glycosides, luteolin, tricin, and chrysoeriol glycosides have been previously separated from alfalfa aerial parts, and their structures have been confirmed by UV, MS, and NMS spectroscopy [5-7]. Some of these compounds were acylated with caffeic, ferulic, sinapic, or coumaric acids. Flavonoid glycosides possess divergent biological activities. These compounds play an important role in plant-insect interactions. Flavonoid glycosides affect insect behavior and performance. Many of them can also modulate the feeding behavior of insects. Our earlier investigations [10] showed that apigenin glycosides modify the behavior of the pea aphid. There was a negative correlation between the concentration of total apigenin glycosides in the alfalfa plants and pea aphid abundance. It was shown that all apigenin glycosides had antifeedant and growth inhibitory effects on the pea aphid. Apigenin glycosides were feeding deterrents in alfalfa plants. There was a negative correlation between pea aphid phloem sap ingestion and the concentration of apigenin glycosides. Although a number of flavonoids from different parts of alfalfa are known, our understanding about their concentrations and effect of the specific flavonoids present in alfalfa on insects is unknown. Nothing has been known about the effects of the levels of luteolin, tricin, and chrysoeriol glycosides from aerial parts of alfalfa on pea aphid.

The function of flavone glycosides in the alfalfa remains unclear. There is very little research into the roles the specific flavonoids have on the behaviour of insects. Because alfalfa is an important crop, both as feed for livestock and as good material for food additive preparations, it seems necessary to characterize its unique flavonoid composition and determine its influence on insects. More research is required to establish the role and bioactivity of alfalfa flavonoid glycosides. The present study, therefore, analyzed luteolin, tricin, and chrysoeriol 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/Blonie (near Warsaw, Poland). Seeds were germinated in an environmental chamber at 21±1°C, with 16 h daylight and 8 h of darkness, and 70% relative humidity. Plants were grown in 7×7×9 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 6-month-old plants that were uninested or infested by *Acyrthosiphon pisum* were used in the experiments.

**Aphids**

The pea aphids came from a stock culture kept at the Siedlce University of Natural Sciences and Humanities, 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±1°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 [12].

**High-Performance Liquid Chromatography of Flavonoids**

Aerial parts of plants that were uninested 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, luteolin glycosides, tricin glycosides, and chrysoeriol followed Oleszek and Stochmal [13]. 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 C18 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 evaporator 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 Stochmal [13]. Flavonoids were separated using a Waters HPLC system, consisting of a model 616 pump and 99 G PAD detector (Waters Corporation, Milford, USA). Millenium 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% H₃PO₄ (65:35%), increasing to 0:100% over 60 min. Chromatograms were registered and integrated at 350 nm. Standards of flavones and their glycosides were purchased from the Biochemical Laboratory Institute of Soil Science and Plant Cultivation (Pulawy, Poland). Standards were prepared according to Stochmal and Oleszek [14]. Stock standard solutions of flavones at concentrations of 1 mg/ml were prepared in MeOH (1, 2) or MeOH-DMso (1:1) and stored at 4°C in darkness. Working solutions were prepared by successive dilutions with MeOH for calibration curve preparation. Chemicals and solvents were of analytical grade. Total flavonoid concentration was calculated from total integration area (350
nm) using the calibration curve of apigenin glycoside (7-O-{2-O-feruloyl-[B-D-GluA-(1-3)-B-D-GluA-(1-2)-O-B-D-Glc]}Apigenin).

Pea Aphid Behavior

Influence of the flavones glycosides on pea aphid behavior (abundance, population parameters, and feeding activities) were analyzed. The observations were carried out using an environmental chamber at 21±1ºC, 16 h daylight and 8 h of darkness, and 70% relative humidity. Plexiglass cages 10×10×30 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. Aphids on each plant were counted after 15 days.

During one generation’s development (from birth to death) the population dynamics of the pea aphid on the alfalfa plants were monitored. The newborn nymphs were counted and removed every day and the mean number of aphids per plant was calculated. Population parameters were determined [15]. The experiments were carried out in 10 independent replicates.

Feeding behaviour of the pea aphid on the Radius cv. was monitored using the Electrical Penetration Graphs (EPG) technique according to Tjallingii [16, 17]. The experiments were run for 8 h for 10 aphids, on 10 different alfalfa 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 behavioral 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).

Statistical Analysis

The differences in levels of the flavonoid glycosides on the studied alfalfa cv. Radius were subjected to one-way ANOVA followed by the post-hoc Newman-Keuls test. The differences in foliar chemistry between uninfested (control) and infested alfalfa plants were analyzed with Student's t test. Influence of the flavones glycosides on pea aphid abundance, population parameters, and feeding activities were analyzed using Spearman rank correlation. The Statistica program for Windows v. 6.0 was used for all statistical analyses [18].

Results

Variation in Flavonoid Profiles and Content among the Studied Alfalfa Plants

Pea aphid uninfested and infested alfalfa plants had similar flavonoid profiles (Fig. 1). Luteolin, tricin, and chrysoeriol glycosides were identified on the basis of the absorption spectra of the chromatograms. Previous HPLC-MS studies have been conducted with these compounds.

<table>
<thead>
<tr>
<th>Substance category</th>
<th>Alfalfa plants</th>
<th>Statistics</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Uninfested</td>
<td>Infested</td>
</tr>
<tr>
<td>Total flavonoids</td>
<td>11.13±0.63</td>
<td>12.34±0.54</td>
</tr>
<tr>
<td>Flavonoids nonacylated</td>
<td>0.77±0.06</td>
<td>0.85±0.07</td>
</tr>
<tr>
<td>Flavonoids acylated</td>
<td>2.78±0.62</td>
<td>3.14±0.26</td>
</tr>
<tr>
<td>Total luteolin glycosides</td>
<td>5.21±0.06</td>
<td>5.21±0.12</td>
</tr>
<tr>
<td>Luteolin nonacylated</td>
<td>1.85±0.03</td>
<td>1.85±0.04</td>
</tr>
<tr>
<td>Luteolin acylated</td>
<td>3.36±0.09</td>
<td>3.37±0.08</td>
</tr>
<tr>
<td>Total tricin glycosides</td>
<td>2.20±0.02</td>
<td>2.21±0.01</td>
</tr>
<tr>
<td>Tricin nonacylated</td>
<td>1.69±0.01</td>
<td>1.68±0.01</td>
</tr>
<tr>
<td>Tricin acylated</td>
<td>0.52±0.01</td>
<td>0.49±0.03</td>
</tr>
<tr>
<td>Total chrysoeriol glycosides</td>
<td>0.14±0.02</td>
<td>0.15±0.01</td>
</tr>
</tbody>
</table>

Values in columns followed by different letters are different at P ≤ 0.05 (Newman-Keuls test).
Compounds 1 and 2 were luteolin glycosides, 8 chrysoeri-
ol and 3, 4, 5, 6, and 7 tricin glycosides. Three compounds
(1, 8, and 3) were nonacylated and the rest were acylated.
Compounds 2, 5, and 6 were acylated with ferulic acid,
compound 4 with sinapic acid, and compound 7 with glu-
curonic acid (Fig. 2).

The total concentration of flavonoids was high but did
not differ significantly between aphid-infested and uninfest-
ed alfalfa plants (Table 1). It was shown that total luteolin
glycosides were the dominant flavonoid glycosides of the
pea aphid infested and uninfested alfalfa plants and chrysoe-
riol glycosides were present in the smallest amounts (Table
1). The total concentration of luteolin, tricin, and chrysoeri-
ol glycosides, non-acylated and acylated individual flavonoid
glycosides also did not differ significantly between aphid-
infested and uninfested alfalfa plants (Table 1).

Flavonoid analyses revealed substantial individual vari-
ation (Table 2). It was shown that (2) compound was the
dominant luteolin glycoside of pea aphid infested and unin-
fested alfalfa plants and compound (3) were present in the
smallest amounts. (Table 2). The concentrations of com-
pound (6) differ significantly between aphid-infested and
uninfested alfalfa plants. There were no statistical differ-
cences in the other events (Table 2).

<table>
<thead>
<tr>
<th>Comp.</th>
<th>R₁</th>
<th>R₂</th>
<th>R₃</th>
<th>R₄</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-OH</td>
<td>-OH</td>
<td>-H</td>
<td>-O-GluA</td>
</tr>
<tr>
<td>2</td>
<td>-OH</td>
<td>-O-GluA</td>
<td>-H</td>
<td>-O-GluA(2→1)GluA-2-O-Feruloyl</td>
</tr>
<tr>
<td>3</td>
<td>-OCH₃</td>
<td>-OH</td>
<td>-OCH₃</td>
<td>-O-GluA(2→1)GluA</td>
</tr>
<tr>
<td>4</td>
<td>-OCH₃</td>
<td>-OH</td>
<td>-OCH₃</td>
<td>-O-GluA(2→1)GluA-2-O-Synapoyl</td>
</tr>
<tr>
<td>5</td>
<td>-OCH₃</td>
<td>-OH</td>
<td>-OCH₃</td>
<td>-O-GluA(2→1)GluA-2-O-Feruloyl</td>
</tr>
<tr>
<td>6</td>
<td>-OCH₃</td>
<td>-OH</td>
<td>-OCH₃</td>
<td>-O-GluA(2→1)GluA(1→3)-2-O-Feruloyl</td>
</tr>
<tr>
<td>7</td>
<td>-OCH₃</td>
<td>-OH</td>
<td>-OCH₃</td>
<td>-O-GluA</td>
</tr>
<tr>
<td>8</td>
<td>-OCH₃</td>
<td>-O-GluA</td>
<td>-H</td>
<td>-O-GluA</td>
</tr>
</tbody>
</table>

Fig. 1. High-performance liquid chromatography analysis of flavonoids from aerial parts of cv. Radius. Peak identities: (1) 7-O-β-D-
glucuronopyranosyl-luteolin; (2) 7-O-[2-O-feruloyl-β-D-glucuronopyranosyl(1→2)-O-β-D-glucuronopyranosyl]-4′-O-β-D-glucuronopy-
ranosyl-luteolin; (3) 7-O-β-D-glucuronopyranosyl(1→2)-O-β-D-glucuronopyranosidetricin; (4) 7-O-[2′-O-sinapoyl-β-D-glucuronopy-
ranosyl(1→2)-O-β-D-glucuronopyranosidetricin; (5) 7-O-[2′-O-feruloyl-β-D-glucuronopyranosyl(1→2)-O-β-D-glucuronopy-
ranosidetricin; (6) 7-O-[2′-O-feruloyl-β-D-glucuronopyranosyl(1→3)]-O-β-D-glucuronopyranosyl(1→2)-O-β-D-glucuronopy-
ranosidetricin; (7) 7-O-β-D-glucuronopyranosidetricin; (8) 7-O-β-D-glucuronopyranosyl-4′-O-β-D-glucuronopy-
ranosidetricin; (9) 7-O-β-D-glucuronopyranosidetricin.

Fig. 2. Chemical formula of analyzed alfalfa flavones.
Effect of Alfalfa Glycosides on Pea Aphid Behavior

The obtained results showed that the number of aphids was rather small (14 aphids/plant) and the development was poor. The pre-reproductive period was long (14 days on average), the daily fecundity only 3-4 nymphs, and the development time of one generation ca. only 19 days.

The electronic registration (EPG) of A. pisum probing behaviour on alfalfa cv. Radius revealed waveform C, which represents probing in mesophyll, and waveforms E1 and E2, which indicate salivation in phloem vessels and ingestion of sap, respectively, and waveform G, which reflects ingestion of xylem sap. Generally, the total probing times was short (Fig. 3). The duration of activity in phloem tissues (E1 and E2) was the lowest. The probes represented stylet activities related only to penetration of non-phloem tissues (C), were longer, and took up ca. 51% of the probing time.

Pea aphid behavior on alfalfa cv. Radius was affected by the studied flavonoid glycosides. A positive correlation was found between the pea aphid abundance and concentration of total nonacylated compounds (rs=0.45, P<0.010), total tricin glycosides (rs=0.47, P<0.010), and total nonacylated tricin glycosides (rs=0.46, P<0.010). Negative correlations were found between pea aphid abundance and the concentration of individual flavonoid glycosides for the remaining events.

Pea aphid daily fecundity per female was correlated by nonacylated flavonoids (rs=0.61, P<0.05), total luteolin glycosides (rs=0.57, P<0.05), luteolin nonacylated glycosides (rs=0.60, P<0.05), luteolin acylated glycosides (rs=0.58, P<0.05), tricin nonacylated glycosides (rs=0.57, P<0.05), and chrysoriol glycosides (rs=0.66, P<0.05). Correlations between pea aphid pre-reproductive period and total tricin glycosides were found (rs=0.64, P<0.05). Correlations between the pre-reproductive period and the content of other tested compounds was not found. Correlation between other studied population parameters (periods: reproductive and post-reproductive, total fecundity, and survival) and the concentration of tested compounds was not found neither.

Correlation between the concentrations of luteolin and tricin glycosides in alfalfa and pea aphid feeding behavior parameters was not found. Statistical analysis proved that the level of the chrysoeriol glycosides was correlated with xylem sap ingestion (rs=0.644, P=0.044). Correlation between the concentration of chrysoeriol glycosides in alfalfa plants and other feeding behavior parameters was not found.

Table 2. The concentration (mg/g dry matter ±SE) of individual flavonoid glycosides for Radius cv infested and uninfested by pea aphid.

<table>
<thead>
<tr>
<th>Flavonoid glycosides*</th>
<th>Alfalfa plants</th>
<th>Statistics</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Uninfested</td>
<td>Infested</td>
</tr>
<tr>
<td>(1)</td>
<td>1.180±0.012</td>
<td>1.20±0.029</td>
</tr>
<tr>
<td>(2)</td>
<td>3.330±0.058</td>
<td>3.360±0.035</td>
</tr>
<tr>
<td>(3)</td>
<td>0.144±0.001</td>
<td>0.146±0.001</td>
</tr>
<tr>
<td>(4)</td>
<td>0.081±0.002</td>
<td>0.090±0.017</td>
</tr>
<tr>
<td>(5)</td>
<td>0.158±0.003</td>
<td>0.160±0.006</td>
</tr>
<tr>
<td>(6)</td>
<td>0.104±0.001</td>
<td>0.106±0.003</td>
</tr>
<tr>
<td>(7)</td>
<td>0.099±0.001</td>
<td>0.145±0.004</td>
</tr>
</tbody>
</table>

Values in columns followed by different letters are different at P ≤ 0.05 (Newman-Keuls test).

* For individual flavonoid glycosides see Fig. 1.

Discussion

To date little is known about alfalfa flavonoids with respect to their chemistry and biological activity, especially in relation to insects. Not much has been known about alfalfas’ luteolin, tricin, and chrysoriol glycosides and their role in plant-insect interactions. Their effect on the A. pisum
aphid has not been extensively studied. In the present study of the aerial parts of alfalfa cv. Radius, eight flavonoids (as luteolin, tricin, and chrysoeriol glycosides) were identified. These glycosides are rare in plants. Some of the above luteolin, tricin, and chrysoeriol structures have been previously reported in alfalfa varieties [18]. The luteolin, tricin, and chrysoeriol 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
[5, 6].

Alfalfa flavones are a mixture of acylated and nonacetylated forms. In our study, we found that both types of these forms grew in similar concentrations in infested alfalfa plants as in uninfested ones. Additionally, the concentration of acylated flavones was higher than non-acylated and acylated luteolin glycosides higher than nonacylated ones. Opposite trends were found for tricin glycosides, and the concentration of non-acylated form was higher than acylated ones. Stochem and Oleszek [18] suggested the acylated forms are more important for the plant than non-acylated ones in alfalfa plant strategy to protect the plant from different damages, especially UV-B radiation. Simmonds [19] showed the structure of compounds, the types of flavonoids could modify insect feeding and development.

Flavonoids have been recognized as active compounds. Birch leaf surface flavonoid aglycones affected the growth rate of the fifth instar and the pupal mass of the most destructive pest of birch, lepidopteran *Epirrita autumnata* [20]. Flavonoid aglycones have also been shown to reduce the growth rate and prolong the duration of the first instar *E. autumnata* larvae [21]. Lahtinen et al. [22] showed significantly negative effects of increased contents of both total flavonoid and individually fed flavonoid compounds for the larval performance of certain mid-to-late and late season, sawfly species. Our experiments showed that the effect of alfalfa on the pea aphid was associated with the level of flavonoids. In our study we showed that pea aphid abundance was correlated with the concentration of total flavonoid glycosides, acylated and non-acylated glycosides, and the concentration of individual flavonoids. The adverse effect of plant phenolics on other aphid species has been reported earlier and abundance of the pea aphid was also affected by these compounds [23]. Agrell et al. [24] reported that the total concentration of flavones in *Spodoptera littoralis* infested and uninfested alfalfa plants were not significantly different. Similar trends were shown by us in this study.

Flavonoids affect aphids’ behavior and performance [10, 25]. It has been shown that apigenin and apigenin glycosides are feeding deterrents to herbivores [24, 26, 27]. Guerin et al. [28] showed that the high mortality of insects was caused by apigenin. Golawska et al. [10] found that apigenin glycosides modify the behavior of the pea aphid. There was a negative correlation between the concentration of total apigenin glycosides in the alfalfa plants and pea aphid abundance and phloem sap ingestion. In our study we found that pea aphid daily fecundity was caused by luteolin, tricin, and chrysoeriol glycosides. Simmonds and Stevenson [29] showed that iso flavonoids from different wild relatives of *Cicer* had antifeedant activity against *Helicoverpa armigera*. The Golawska and Lukasik [23] study showed that peripheral tissue penetration by *A. pismum* was negatively affected by total phenolics. Bouaziz et al. [30] showed that flavonoids (tricin, tricin 7-O-glucoside) from *Hyparrhenia hirta* Stapf. (Poaceae) stimulated the feeding of the locusts *Locusta migratoria* and *S. gregaria*, but not *Spodoptera frugiperda*. One more flavonoid tested, luteolin, did not influence the feeding of all three species of insects. In this study we showed that pea aphid feeding behaviour on alfalfa cv. Radius was not affected by luteolin and tricin glycosides, but we showed that chrysoeriol modulated a xylem sap ingestion.

Our earlier data showed that alfalfa plants affected the behavior of the pea aphids [8, 23, 24]. Results presented here suggest that the flavonoid compounds affected pea aphid fecundity and imply that cv. Radius are not good hosts for the pea aphid. The function of flavones in the host plant, how alfalfa is, remains unclear and their nutritional implications are also uncertain. Further research is required to establish the role and activity of these compounds in alfalfa plants. A systematic effort is needed to clarify the flavonoid roles together with the other secondary metabolites especially saponins, which have been widely widespread and showed biological activity in alfalfa and primary nutrients in insect behavior. Studies into these interactions are needed to better understand how a group of compounds like flavonoids in plants could influence insect behavior.

**Acknowledgements**

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**References**
