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*Original Research*

# Do the Contents of Luteolin, Tricin, and Chrysoeriol Glycosides in Alfalfa (*Medicago sativa* L.) Affect the Behavior of Pea Aphid (*Acyrthosiphon pisum*)?

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**Abstract**

Flavonoids play an important role in interactions of plants with the environment. Liquid chromatography (HPLC) was used to determine the flavonoid profiles (especially luteolin, tricin, and chrysoeriol glycosides), their total concentrations, and changes in the amounts of eight flavones found in the aerial parts of alfalfa (*Medicago sativa* L.) (Fabaceae) Radius cv., uninfested and infested by the pea aphid (*Acyrthosiphon pisum* Harris) (Homoptera: Aphididae). It was 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→2)-O-*β*-D-glucuronopyranosyl]-4'-O-*β*-D-glucuronopyranosideluteolin. Compound 7-O-{2'-O-feruloyl-[*β*-D-glucuronopyranosyl(1→3)]-O-*β*-D- glucuronopyranosyl(1→2)-O-*β*-D- glucuronopyranosidetricin was present in the smallest amounts. The total concentration of flavones was rather high and ranged from 11.13 to 12.34 mg/g d.m., but there were no significant differences between uninfested and infested alfalfa plants. There was a correlation between the concentration of flavonoid glycosides in the alfalfa plants and pea aphid abundance. Pea aphid daily fecundity per female was affected by luteolin, tricin, and chrysoeriol glycosides and the level of chrysoeriol glycosides affected ingestion of xylem sap by the aphid. This finding may indicate that the studied flavonoid glycoside forms are biologically active in alfalfa – *A. pisum* interactions.

**Keywords:** *Medicago sativa*, flavonoid glycosides, HPLC, *Acyrthosiphon pisum*

## 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 (Homoptera: 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

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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/Błonie (near Warsaw, Poland). Seeds were germinated in an environmental chamber at 21±1ºC, 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 uninfested 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±1ºC, 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 uninfested 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 40ºC 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 40ºC 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 [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% $H_3PO_4$ (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 (Puławy, 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) (3-8) and stored at 4ºC 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

Table 1. Foliar chemistry (mg/ g dry matter ±SE) for Radius cv infested and uninfested by pea aphid.

| Substance category | Alfalfa plants | | Statistics | |
|---|---|---|---|---|
| | Uninfested | Infested | t | P-value |
| Total flavonoids | $11.13^{a}$±0.63 | $12.34^{a}$±0.54 | 1.464 | 0.217 |
| Flavonoids nonacylated | $0.77^{f}$±0.06 | $0.85^{e}$±0.07 | 1.187 | 0.280 |
| Flavonoids acylated | $2.78^{cd}$±0.62 | $3.14^{c}$±0.26 | 0.545 | 0.614 |
| Total luteolin glycosides | $5.21^{b}$±0.06 | $5.21^{b}$±0.12 | -0.023 | 0.982 |
| Luteolin nonacylated | $1.85^{e}$±0.03 | $1.85^{d}$±0.04 | 0.151 | 0.887 |
| Luteolin acylated | $3.36^{c}$±0.09 | $3.37^{c}$±0.08 | -0.084 | 0.937 |
| Total tricin glycosides | $2.20^{de}$±0.02 | $2.21^{d}$±0.01 | -0.541 | 0.617 |
| Tricin nonacylated | $1.69^{e}$±0.01 | $1.68^{d}$±0.01 | 0.630 | 0.563 |
| Tricin acylated | $0.52^{f}$±0.01 | $0.49^{ef}$±0.03 | 0.819 | 0.459 |
| Total chrysoeriol glycosides | $0.14^{f}$±0.02 | $0.15^{f}$±0.01 | -2.191 | 0.094 |

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

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.

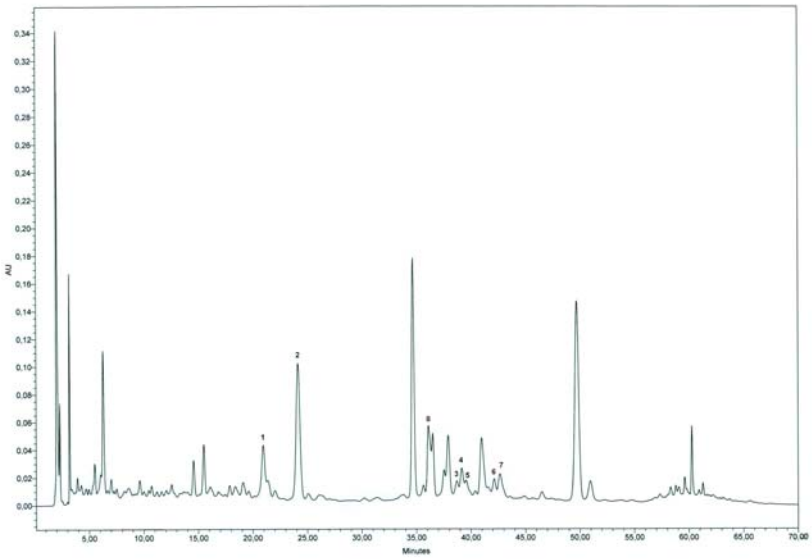
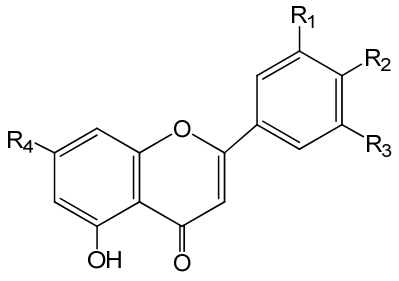


Fig. 1. High-performance liquid chromatography analysis of flavonoids from aerial parts of cv. Radius. Peak identities: (1) 7-O-β-D-glucuronopyranosideluteolin; (2) 7-O-[2-O-feruloyl-β-D-glucuronopyranosyl(1→2)-O-β-D-glucuronopyranosyl]-4'-O-β-D-glucuronopyranosideluteolin; (3) 7-O-[β-D-glucuronopyranosyl(1→2)-O-β-D- glucuronopyranosidetricin; (4) 7-O-[2'-O-sinapoyl-β-D-glucuronopyranosyl(1→2)-O-β-D-glucuronopyranosidetricin; (5) 7-O-[2'-O-feruloyl-β-D-glucuronopyranosyl(1→2)-O-β-D-glucuronopyranosidetricin; (6) 7-O-{2'-O-feruloyl-[β-D-glucuronopyranosyl(1→3)]-O-β-D- glucuronopyranosyl(1→2)-O-β-D- glucuronopyranosidetricin; (7) 7-O-β-D-glucuronopyranosidetricin; (8) 7-O-β-D-glucuronopyranosyl-4'-O-β-D-glucuronopyranosidechrysoeriol.

Compounds 1 and 2 were luteolin glycosides, 8 chrysoeriol 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 glucuronic acid (Fig. 2).

The total concentration of flavonoids was high but did not differ significantly between aphid-infested and uninfested 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 chrysoeriol glycosides were present in the smallest amounts (Table 1). The total concentration of luteolin, tricin, and chrysoeriol 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 variation (Table 2). It was shown that (2) compound was the dominant luteolin glycoside of pea aphid infested and uninfested alfalfa plants and compound (3) were present in the smallest amounts. (Table 2). The concentrations of compound (6) differ significantly between aphid-infested and uninfested alfalfa plants. There were no statistical differences in the other events (Table 2).




| Comp. | $R_1$ | $R_2$ | $R_3$ | $R_4$ |
|---|---|---|---|---|
| 1 | -OH | -OH | -H | -OGluA |
| 2 | -OH | -OGluA | -H | -OGluA(2→1)GluA-2-*O*-Feruloyl |
| 3 | $-OCH_3$ | -OH | $-OCH_3$ | -OGluA(2→1)GluA |
| 4 | $-OCH_3$ | -OH | $-OCH_3$ | -OGluA(2→1)GluA-2-*O*- Synapoyl |
| 5 | $-OCH_3$ | -OH | $-OCH_3$ | -OGluA(2→1)GluA-2-*O*-Feruloyl |
| 6 | $-OCH_3$ | -OH | $-OCH_3$ | -OGluA(2→1)GluA[GluA(1→3)]-2-*O*-Feruloyl |
| 7 | $-OCH_3$ | -OH | $-OCH_3$ | -OGluA |
| 8 | $-OCH_3$ | -OGluA | -H | -OGluA |

Fig. 2. Chemical formula of analyzed alfalfa flavones.

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

| Flavonoid glycosides* | Alfalfa plants | | Statistics | |
|---|---|---|---|---|
| | Unifested | Infested | t | P-value |
| (1) | 1.180$^{b}$±0.012 | 1.20$^{b}$±0.029 | -0.643 | 0.5550 |
| (2) | 3.330$^{a}$±0.058 | 3.360$^{a}$±0.035 | -0.446 | 0.6789 |
| (8) | 0.144$^{cd}$±0.001 | 0.146$^{c}$±0.001 | -1.000 | 0.3739 |
| (3) | 0.081$^{c}$±0.002 | 0.090$^{d}$±0.017 | -0.517 | 0.6324 |
| (4) | 0.158$^{c}$±0.003 | 0.160$^{c}$±0.006 | -0.297 | 0.7812 |
| (5) | 0.104$^{cd}$±0.001 | 0.106$^{cd}$±0.003 | -0.643 | 0.5550 |
| (6) | 0.099$^{cd}$±0.001 | 0.145$^{c}$±0.004 | -10.944 | 0.0004 |
| (7) | 0.140$^{cd}$±0.017 | 0.160$^{c}$±0.002 | -1.144 | 0.3162 |

Values in columns followed by different letters are different at $P \leq 0.05$ (Newman-Keuls test).
* For individual flavonoid glycosides see Fig. 1.

## 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 ($r_s$=0.45, P<0.010), total tricin glycosides ($r_s$=0.47, P<0.010), and total nonacylated tricin glycosides ($r_s$=0.46, P<0.010). Negative correlations were found between pea aphid abundance and the concentration of individual flavonoid glycosides for the remaining events.

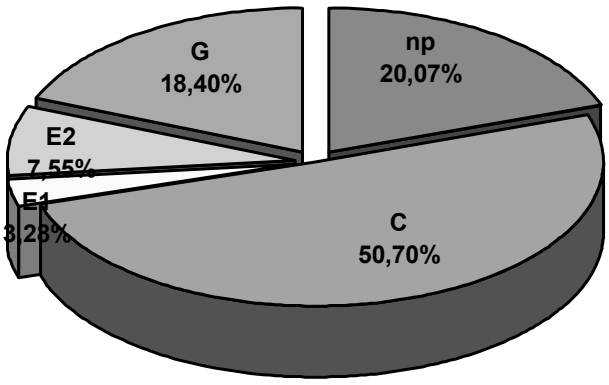



Fig. 3. *A. pisum* aphid activity while probing tissues of alfalfa cv Radius, during 8 hours of EPG recordings; np – no probing; C – pathways; E1 – salivation into sieve elements; E2 – phloem sap ingestion; G – xylem sap ingestion.

Pea aphid daily fecundity per female was correlated by nonacylated flavonoids ($r_s$=0.61, P<0.05), total luteolin glycosides ($r_s$=0.57, P<0.05), luteolin nonacylated glycosides ($r_s$=0.60, P<0.05), luteolin acylated glycosides ($r_s$=0.58, P<0.05), tricin nonacylated glycosides ($r_s$=0.57, P<0.05), and chrysoriol glycosides ($r_s$=0.66, P<0.05). Correlations between pea aphid pre-reproductive period and total tricin glycosides were found ($r_s$=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 ($r_s$=-0.644, P=0.044). Correlation between the concentration of chrysoeriol glycosides in alfalfa plants and other feeding behavior parameters was not found.

## 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 chrysoeriol 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 non-acylated forms. In our study, we found that both types of these forms grew in similar concentrations in infested alfalfa plants as in unifested ones. Additionally, the concentration of acylated flavones was higher than non-acylated and acylated luteolin glycosides higher than non-acylated ones. Opposite trends were found for tricin glycosides, and the concentration of non-acylated form was higher than acylated ones. Stochmal 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.

Falvonoids 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 unifested 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. Goławska 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 isoflavonoids from different wild relatives of *Cicer* had antifeedant activity against *Helicoverpa armigera*. The Goławska and Łukasik [23] study showed that peripheral tissue penetration by *A. pisum* 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.

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