

## RELATIONSHIP BETWEEN SAPONIN CONTENT IN ALFALFA AND APHID DEVELOPMENT

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We studied the effect of qualitative and quantitative variation of saponin content in foliar tissues of four European alfalfa (*Medicago sativa* L.) cultivars (Radius, Sapko, Sitel, Radius line 1) on pea aphid (*Acyrtosiphon pisum* Harris) development, and the effect of aphid infestation on alfalfa saponin content. Aphids (adult apterae, larvae, and adult alatae) were counted on 3-, 6- and 9-month-old plants (before the first, second and third cutting). Thin-layer chromatography was used to detect and estimate the quantity of the following saponins: 3GlcA, 28AraRhaXyl medicagenic acid; 3Glc, 23Ara, 28AraRhaXylApi zanhic acid (zanhic acid tridesmoside); and 3RhaGalGlcA soyasapogenol B (soyasaponin I). Radius, Sapko, and Sitel contained all three saponins but Radius line 1 did not contain zanhic acid tridesmoside or medicagenic acid glycoside. Saponin content was highest in Radius and lowest in Radius line 1. Regardless of the cultivar, saponin content was higher in aphid-infested than uninfested plants. For all sampling dates, aphid numbers were highest on Radius line 1 and lowest on Radius; that is, aphid numbers were inversely related to saponin content. Alfalfa has a herbivore-induced defense. Saponin levels increase in the foliage of infested alfalfa. Attempts of plant breeders to reduce saponin content in order to increase alfalfa digestibility for livestock might make the plants more susceptible to aphids and other pests.

**Key words:** Saponins, *Medicago sativa*, *Acyrtosiphon pisum*.

### INTRODUCTION

Alfalfa (*Medicago sativa* L.) (Fabaceae) is the most important livestock feed crop (Small, 1996) and has great potential as food and fodder. It has been used as feed for livestock in the form of green feed, hay and pellets. Alfalfa sprouts are widely consumed as a garnish, and leaf protein concentrates and the dehydrated plant are components of many nutritional supplements (Stochmal et al., 2001a). The high nutritional quality of alfalfa results from its substantial content of high quality protein and carbohydrates (Hegsted and Linkswiler, 1980; Hatfield, 1992). Aerial parts of the plant are sources of chlorophyll, vitamins, some digestive enzymes and  $\beta$ -carotene (Duke, 1992; Goławska et al., 2010a).

Alfalfa can be seriously damaged by pests, including the pea aphid (*Acyrtosiphon pisum* Harris 1776). Previous research demonstrated a reduction of alfalfa yields as a function of pea aphid

abundance (Cuperus et al., 1982). The pea aphid is also an important vector of legume viruses (Müller, 1971).

In addition to the nutritional components that make alfalfa useful as an animal feed or food supplement (Hatfield, 1992), the plants produce a variety of secondary metabolites showing biological activity. Many of these compounds help protect the plant against herbivores (Cambier et al., 2000; Awmack and Leather, 2002) and can influence the choice of food sources by insect herbivores (Shonle and Bergelson, 2000; Lankau, 2007; Mosleh et al., 2008).

The many kinds of secondary metabolites in alfalfa (Oleszek et al., 1992; Stochmal et al., 2001a,b; Goławska et al., 2010b) include saponins (Livingston et al., 1980; Oleszek et al., 1992). Alfalfa has relatively high levels of saponins. Saponin concentrations in different alfalfa varieties range from 0.8% to 2.0% (Pedersen and Wang, 1971; Majak et

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al., 1980). Alfalfa cultivars differ in the content of saponins such as medicagenic acid, zanhic acid, and soyasapogenol glycosides (Kapusta et al., 2005a; Pecetti et al., 2006).

Saponins possess anti-inflammatory, hemolytic, cholesterol lowering and anticancer properties (Oleszek, 1990; Jurzysta and Waller, 1996). These compounds have also been associated with allelopathy, poor digestibility in ruminants, inhibition of enzyme activity, deterrence of insect foraging, and antifungal effects (Tava and Odoardi, 1996; Kocacaliskan et al., 2009). Saponins have been suggested as possible chemical defensive agents against generalist herbivores (Oleszek et al., 1990; Nozzolillo et al., 1997; Osbourn, 2003). The presence of saponins in alfalfa used for forage is considered undesirable, however, because saponins act as antinutritional factors in livestock. They inhibit weight gain in young animals and depress egg production in hens (Price et al., 1987). Agrell et al. (2003) found strong evidence that saponins are involved in the herbivore-induced defense of alfalfa. Saponin levels were higher in damaged than in control plants.

A previous study showed that saponins are toxic to the pea aphid and may act in resistance against it (Goławska, 2007). Here we sought to determine whether the presence and relative amount of saponins in foliar tissues of four European alfalfa cultivars were related to the number of pea aphids infesting them.

## MATERIALS AND METHODS

### PLANT MATERIAL

We used three European cultivars of alfalfa (*Medicago sativa*) (Radius, Sapko, Sitel), and one line of Radius (see Goławska and Łukasik, 2009) herein called "Radius line 1." Sitel seeds were purchased from Horticultural Plant Breeding, Seed Production and Nursery in Ożarów Mazowiecki (Warsaw, Poland), and the other seeds were obtained from the Plant Breeding and Acclimatization Institute (IHAR) in Radzików/Błonie (near Warsaw, Poland). Seeds were germinated and grown in a climate chamber kept at 21°C with 70% relative humidity (RH) and a 16 h photoperiod. The plants were grown in plastic pots (7 × 7 × 9 cm) containing garden soil, one plant per pot. The plants were watered regularly but not fertilized.

### APHIDS

The pea aphids (*Acyrtosiphon pisum* Harris) used in the experiment (see next section) were obtained from a stock culture kept at the University of

Natural Sciences and Humanities in Siedlce, Poland. The aphids were reared on seedlings of *Vicia faba* L. var. Start (Fabaceae) (broad bean) in an environmental chamber (21±1°C, 16 h photoperiod, 70% RH). They were maintained on the four alfalfa cultivars for one generation (Apablaza and Robinson, 1967) before the adult apterous females were used in the experiment.

### EXPERIMENT

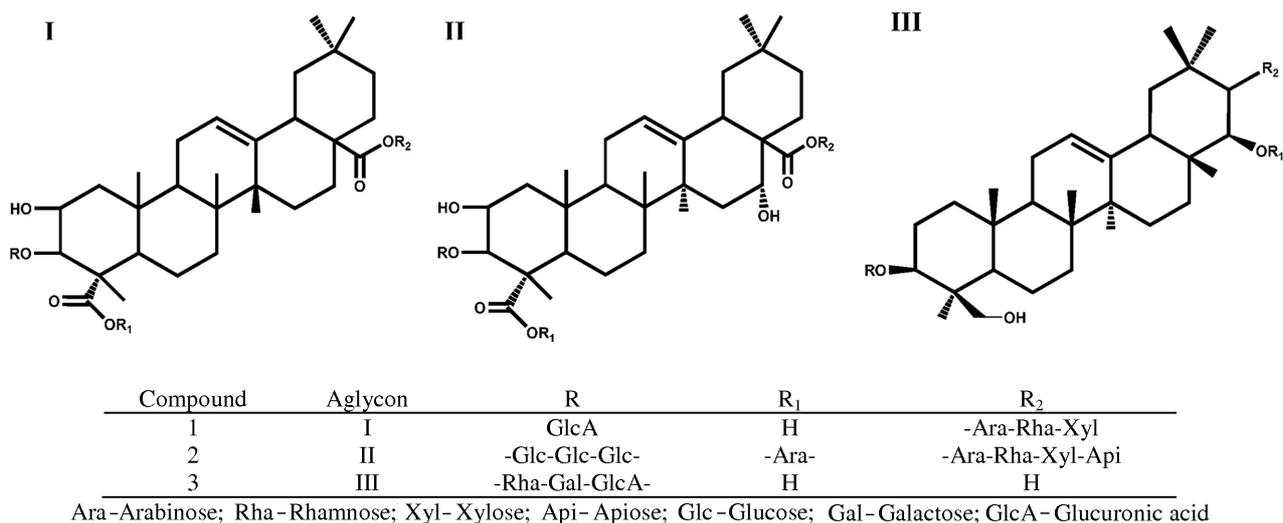
When the alfalfa plants were 2 months old, 20 plants of each cultivar were placed in plastic cylinders (50 × 50 × 50 cm) with mesh covers for ventilation, one plant per cylinder. For each cultivar, 10 plants were infested with 25 adult apterous females, and 10 plants were left uninfested as controls.

The aerial parts of the alfalfa plants were harvested when the plants were 3, 6 and 9 months old (i.e., the first, second and third cuttings). The harvested tissues were freeze-dried, ground, and kept in a desiccator in darkness until their saponin content was assessed by TLC (see next section). The aphids (adult apterae, larvae, and adult alatae) on each plant were counted just before the first, second and third cuttings at months 3, 6 and 9.

### EXTRACTION AND TLC OF SAPONINS

Alfalfa saponins were analyzed according to Oleszek and Stochmal (2002). A 0.5 g sample of the freeze-dried material of each alfalfa cultivar (from 10 plants combined to form one sample) was extracted for 20 min with 70% methanol using an ASE 200 accelerated solvent extractor (Dionex Corporation, Sunnyvale, U.S.A.). The extracts were concentrated at 40°C on a rotary evaporator until the methanol was removed and then were loaded on C18 cartridges (Waters, Poland) preconditioned with water. The saponins were washed from the cartridges with water and 80% methanol. Methanolic fractions were evaporated on a rotary evaporator at 40°C, redissolved in 1 ml 80% MeOH, and used for TLC determination.

The saponins were chromatographed on silica gel (Kieselgel 60 F254) precoated Merck plates. Separation was done using a mobile phase (ethyl acetate-acetic acid-water, 7:2:2 v/v/v). Saponins were detected with modified Liebermann-Burchard's reagent (mixture of sulphuric acid and methanol, 1:5 v/v). After the plates were sprayed with the reagent, they were dried and heated at 105°C for visualization. Sprayed plates were observed with ultraviolet illumination (300 nm). The saponins were identified by comparison with authentic standards purchased from the Biochemical Laboratory, Institute of Soil Science and Plant Cultivation (Puławy, Poland) and prepared as described previ-



**Fig. 1.** Chemical formulas of the identified compounds, I – medicagenic acid; II – zanhic acid; III – soyasapogenol B.

ously (Oleszek et al., 1992). For TLC identification we used three previously purified and identified saponins from *M. sativa*: medicagenic acid saponin, zanhic acid tridesmoside, and soyasaponin I. The relative quantity of a particular saponin was compared between cultivars by visually assessing the spot sizes on the TLC plates.

#### STATISTICAL ANALYSIS

The effect of alfalfa cultivar on number of aphids per plant was assessed by one-way ANOVA followed by the post-hoc Newman-Keuls test, using Statistica for Windows v. 6.0 (StatSof, 2003).

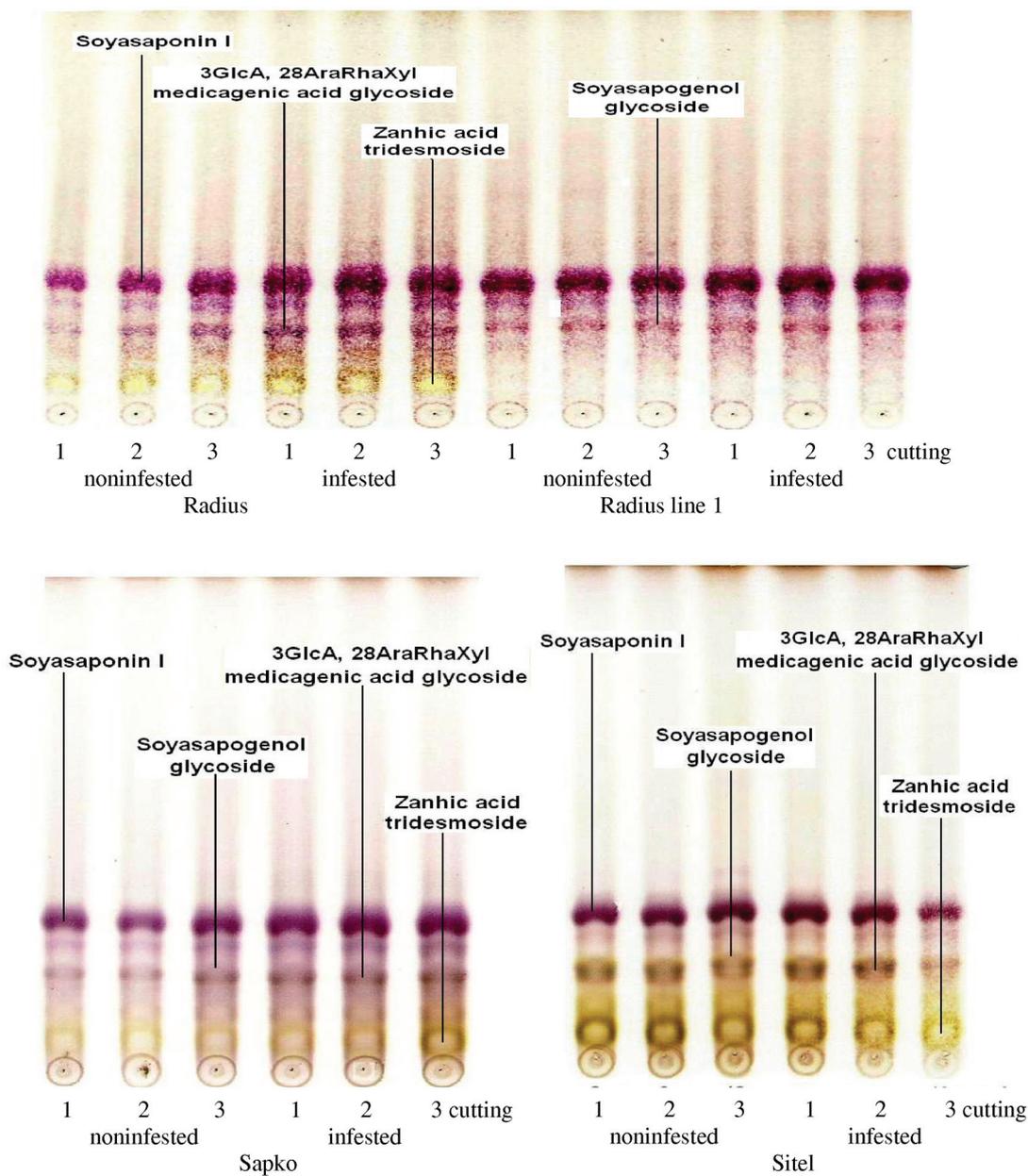
#### RESULTS

The following saponins were detected by TLC separation of aerial tissue extracts: (1) 3GlcA, 28AraRhaXyl medicagenic acid ( $R_f = 0.21$ ), (2) 3Glc, 23Ara, 28AraRhaXylApi zanhic acid (zanhic acid tridesmoside) ( $R_f = 0.07$ ), (3) 3RhaGalGlcA soyasapogenol B (soyasaponin I) ( $R_f = 0.28$ ) and (4) soyasapogenol glycoside with an unidentified aglycone (Fig. 1). Regardless of aphid infestation, the chemical composition of saponins differed between the alfalfa cultivars (Fig. 2). Radius, Sapko and Sitel contained all three saponins. Radius did not contain a fourth compound, soyasapogenol glycoside, which was detected in Radius line 1. Radius line 1 did not contain zanhic acid tridesmoside or medicagenic acid glycoside. These differences were evident at all three cuttings.

As indicated by the intensity of spots on the TLC plates, the saponin concentrations also differed between the alfalfa cultivars. Regardless of the degree of aphid infestation and plant age, the saponin concentration was highest in Radius and lowest in Radius line 1 (Fig. 2). Saponin content increased with plant age. In most cases the saponin concentration was greater in aphid-infested than in uninfested plants (Fig. 2).

Pea aphid abundance (total for all stages) differed between the alfalfa cultivars at the first ( $F_{3,36} = 50.51$ ,  $p < 0.001$ ), second ( $F_{3,36} = 22.55$ ,  $p < 0.001$ ) and third ( $F_{3,36} = 29.80$ ,  $p < 0.001$ ) sampling dates. For all sampling dates, aphid numbers were highest for Radius line 1, lowest for Radius, and intermediate for the two other cultivars (Tab. 1). The number of adult apterae differed between the cultivars at the first ( $F_{3,36} = 37.91$ ,  $p < 0.001$ ), second ( $F_{3,36} = 9.30$ ,  $p < 0.001$ ) and third ( $F_{3,36} = 9.39$ ,  $p < 0.001$ ) sampling dates. The number of larvae differed between the cultivars at the second ( $F_{3,36} = 3.03$ ,  $p < 0.05$ ) and third ( $F_{3,36} = 10.73$ ,  $p < 0.001$ ) sampling dates. The number of adult alatae differed between cultivars at the first ( $F_{3,36} = 7.09$ ,  $p < 0.001$ ), second ( $F_{3,36} = 7.82$ ,  $p < 0.001$ ) and third ( $F_{3,36} = 7.68$ ,  $p < 0.001$ ) sampling dates. For all sampling dates, Radius line 1 supported the highest number of wingless adults and larvae and the lowest number of winged forms (post-hoc Newman-Keuls test,  $p < 0.05$ ) (Tab. 1).

Pea aphid numbers were related to saponin content in that the numbers were highest on the cultivar with the lowest saponin content (Radius line 1), lowest on the cultivar with the highest saponin con-



**Fig. 2.** TLC separation of saponins in four cultivars of alfalfa plants infested by the pea aphid or uninfested. Aphids were applied when the plants were 2 months old, and alfalfa tissue was cut and sampled at 3, 6, and 9 months of growth (sampling times indicated as 1, 2, 3). Each combination of cultivar, sampling time and aphid infestation represented 10 plants and 10 TLC plates; representative TLC plates are shown.

tent (Radius), and intermediate on the two cultivars with intermediate saponin content.

Pea aphid winged forms were related to saponin content in that the numbers were highest on the cultivar with the highest saponin content (Radius), lowest on the cultivar with the lowest saponin content (Radius line 1), and intermediate on the two cultivars with intermediate saponin content.

## DISCUSSION

Our results suggest that saponins are important in the interactions between alfalfa plants and pea aphids: the presence and relative quantity of saponins in alfalfa plants were correlated with differences in pea aphid abundance and development. In a previous study, aphids generally preferred to

TABLE 1. Numbers of pea aphid morphs per plant for four alfalfa cultivars\*

Alfalfa cultivar	Cutting											
	1				2				3			
	Adult apterae	Larvae	Adult alatae	Σ	Adult apterae	Larvae	Adult alatae	Σ	Adult apterae	Larvae	Adult alatae	Σ
Radius	3.3± 0.71c	4.4± 0.70a	5.8± 1.41a	13.5± 1.17c	6.0± 1.32b	9.0± 1.89b	4.4± 1.43a	19.4± 2.27c	7.7± 1.45b	12.1± 1.44c	4.1± 0.57a	23.9± 1.59c
Sapko	8.1± 1.41b	13.4± 1.40a	4.6± 1.41a	26.1± 3.07ab	7.0± 1.76b	10.9± 1.91ab	4.0± 0.47a	22.0± 1.49b	9.3± 1.70b	14.0± 1.41b	3.5± 0.71ab	26.8± 1.75b
Sitel	8.8± 0.70b	10.8± 2.82a	4.8± 1.41a	24.4± 3.19b	6.6± 1.43b	9.5± 2.55ab	3.7± 1.25a	19.9± 1.73c	9.8± 2.78b	12.2± 1.32c	2.7± 0.82bc	24.7± 1.94c
Radius line 1	12.0± 3.53a	12.9± 2.12a	2.8± 0.70b	27.7± 3.40a	10.4± 2.74a	12.4± 3.51a	2.4± 1.29b	25.2± 1.39a	12.5± 1.96a	15.6± 2.11a	2.0± 1.69c	30.3± 1.25a

\*Values are means (± SD) of 10 plants. Values with different letters within columns differ significantly by the Newman-Keuls test at p<0.05.

feed on plants having relatively low amounts of saponins (Goławska and Łukasik, 2009). These results can be explained by the negative effects of saponins on aphid behavior, physiology and metabolism (Goławska, 2007; Goławska et al., 2006, 2008).

The saponins we detected have been reported and identified in alfalfa before, and their structures have been established based on spectral data (Oleszek et al., 1992; Biały et al., 1999). Alfalfa cultivars can differ widely in the kinds of saponins they produce (Tava and Odoardi, 1996; Tava et al., 1999). Our detection of saponins is consistent with previous reports that the three dominant groups of saponins in *Medicago* species are zanhic acid, medicagenic acid and soyasapogenol glycosides (Nowacka and Oleszek, 1994; Kapusta et al., 2005a,b). The specific saponins we detected have been recognized as the dominant saponins in aerial parts of *Medicago* species (Oleszek et al., 1992; Jurzysta and Waller, 1996; Sen et al., 1998; Biały et al., 1999; Achnine et al., 2005; Huhman et al., 2005). Our results on saponin concentrations also are consistent with previous reports that saponin concentrations are high in Radius, intermediate in Sapko and Sitel, and low in Radius line 1 (Staszewski et al., 1994; Goławska and Łukasik, 2009).

Saponins, which have been associated with a wide range of biological properties (Sen et al., 1998; Oleszek, 1990; Wu and Yang, 2004), have been suggested as possible chemical defensive agents against generalist herbivores (Oleszek et al., 1990; Nozzolillo et al., 1997; Osbourn 2003; Agrell et al., 2004). Previous investigations of saponins isolated from *Medicago sativa* have shown that they possess anti-insect activity (Tava and Odoardi, 1996; Agrell et al., 2003). Researchers have suggested that

saponins are toxic to the pea aphid and might act as resistance factors against this species (Kain and Biggs, 1980; Adel et al., 2000; Simmonds, 2001; Goławska, 2007). De Geyter et al. (2007) showed that saponins inhibited growth and even caused mortality in *Acyrtosiphon pisum*. Aphids that feed on plants containing high levels of saponins presumably expend substantial resources on detoxification, which reduces their growth and development. Our results also suggest that the saponin composition in alfalfa affects pea aphid development. The number of the pea aphids was highest for Radius line 1, which contained low levels of saponins, and was lowest on Radius, which contained high levels of saponins. The other two cultivars contained intermediate levels of saponins and supported intermediate numbers of pea aphids.

Many reports have documented the major role of adult apterae in population increase. This morph is involved in intensive exploitation of hosts, reducing their content of the main nutritive compounds. The decrease in host nutrition may stimulate the development of winged morphs (Awmack and Leather, 2002), rejection of the host plant, and migration to more suitable hosts (Vargas et al., 2005; Angeli and Simoni, 2006). In this study the number of *A. pisum* winged morphs was highest on Radius, which also contained the highest concentration of saponins. This correlation suggests that the high saponin content was unfavorable to the pea aphid and probably triggered the production of winged forms.

Our findings also suggest that some saponins are more important than others in the interactions between pea aphids and alfalfa plants. The differences in saponin amounts between the cultivars under aphid attack were greatest for zanhic acid and medicagenic acid glycosides. The levels of 3G1cA,

28AraRhaXyl medicagenic acid glycoside and zanhic acid tridesmoside were higher in Radius plants than in the other three cultivars. In alfalfa these saponins may have higher biological activity than the others against the pea aphid. In addition to acting as deterrents, these compounds may reduce the quality and quantity of the ingested food, reducing fecundity and slowing the growth of the aphid population on Radius. Agrell et al. (2003) reported that medicagenic acids are associated with repelling of insects. Also consistent with this view are reports that the level of 3GlcA, 28AraRhaXyl medicagenic acid doubled in insect-damaged foliage of alfalfa (Agrell et al., 2003) and that 3GlcA, 28AraRhaXyl medicagenic acid is the predominant biologically active saponin in aerial parts of alfalfa, as indicated by assays with the fungus *Trichoderma viride* (Sen et al., 1998). Adel et al. (2000) reported that saponins reduced food consumption and growth of larvae of the moth *Spodoptera littoralis*.

The composition and quantity of chemical compounds in consumed food greatly affect aphid growth and development (Douglas, 2006; Eleftherianos et al., 2006). Aphids decide whether to accept or reject a host plant largely during probing (Ban et al., 2008). The electrical penetration graphs (EPGs) of pea aphid probing of alfalfa tissues containing different levels of saponins corresponded with differences in pea aphid feeding behavior (Goławska et al., 2008). Pea aphids fed much better on Sapko than on Radius. Salivation was longer and ingestion of phloem sap was greater on hosts without deterrent factors than on hosts with deterrent factors (Tjallingii, 1994). In our study the cultivar Radius probably interfered with the feeding activity and thereby lowered the fecundity of *A. pisum*. Using EPGs, Goławska (2007) detected clear differences in pea aphid probing of sucrose-agarose gels containing different concentrations of saponins: the three main alfalfa saponins (zanhic acid tridesmoside; 3GlcA, 28AraRhaXyl medicagenic acid glycoside; 3GlcA, 28AraRha medicagenic acid glycoside) inhibited *A. pisum* feeding. Higher concentrations of these saponins reduced aphid activities that corresponded with ingestion of phloem sap. Saponins derived from medicagenic acid are reported to be the most abundant saponins in alfalfa (Tava et al., 1999).

This study also demonstrated that alfalfa has a herbivore-induced defense. Levels of saponins were higher in aphid-infested than in uninfested alfalfa plants. The induced defense of alfalfa affected feeding behavior and had negative effects on pea aphid development. Our results are in accord with previous studies (Agrawal et al., 1999; Adel et al., 2001; Agrell et al., 2003).

The major saponins in the aerial parts of the four alfalfa cultivars we studied are zanhic and med-

icagenic acid glycosides. The levels of these compounds affect pea aphid biology. These saponins of alfalfa act as natural barriers to feeding by insects. Reducing their content in alfalfa tissue through breeding programs may produce a crop that is susceptible to generalist, well-established pests. Attempts by plant breeders to reduce saponin content in order to increase alfalfa's digestibility for livestock might simultaneously make them more susceptible to aphids and other pests. The plant breeder who selects to minimize digestive problems in domestic animals and humans may also be reducing the plant's natural resistance to herbivores. It may be undesirable from the point of view of pest control. The breeder must weigh improved nutritional quality against potentially lower yields.

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