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Alfalfa (*Medicago sativa* L.) shoot saponins: identification and bio-activity by the assessment of aphid feeding

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Abstract

Biochemical components in alfalfa (*Medicago sativa* L.), such as saponins, can act as protecting factors against bio-stresses. Saponins are also antifeedants and show oral toxicity towards higher and lower animals. Changes in saponins, such as variation in the carbon skeleton, or hydrolysis of saponin glycosides and other conjugates, may change their biological effects. The aims of this research were to study saponin variation in different growth stages of alfalfa and to investigate the biological role of saponins in the spotted alfalfa aphid, *Therioaphis maculata*. Saponins from alfalfa shoots in different growth stages were extracted, chemically purified and analysed by TLC. Specific saponins such as soyasaponin1 from root and shoot and two bisdesmosides of medicagenic acid, one from shoot and another from root tissues, were identified using reference compounds allowing changes in saponin composition during plant development in different shoot tissues of alfalfa to be assessed. The response of the alfalfa aphid to feeding on alfalfa in different growth stages was studied. No significant difference in the survival of aphids, from neonate to adult, was observed, but due to the antibiotic effects of saponins, two differences were found in the onset of nymph production and cumulative nymph production. The results show that the saponin composition in alfalfa changes with plant development and this, in turn, can often negatively affect the development of specific insect pests such as the spotted alfalfa aphid, suggesting a possible biological role of alfalfa saponins.

Key words: Alfalfa shoot saponins: Plant development: Aphid feeding assessment: Biological effects

Saponins, non-protein amino acids, polysaccharides and proteins like lectins and enzyme inhibitors act as plant protection factors[1]. Among these compounds, alfalfa has relatively high levels of saponins. Saponins, named after their foam-producing properties[2,3], are widely distributed in plants, including some foods such as beans, soyabeans, peas, spinach, tomatoes, potatoes, onions, garlic, alliums, asparagus and other plants like alfalfa (*Medicago sativa* L.). The kind and amount of saponins are different in each species[2,4–6]. Plant saponins can be orally toxic towards animals when present in large amounts. Alfalfa saponins were also found to be nutritionally undesirable in poultry, rats, rabbits and swine[7–10]. These compounds have been assumed to be degradable by rumen microorganisms and exert little biological activity in ruminants[11]. The effects of saponins on snails, fungi, viruses, protozoa, rat hepatoma cells, malignant cells, fish respiratory epithelia, cell membranes, animal growth and feed intake, nutrient uptake, protein digestion, oxidation reactions, cholesterol metabolism, animal reproduction, immune system and nervous system have been investigated. The mentioned activities have been reviewed as the biological roles of saponins in birds, animals – even in single-stomached animals – and cold-blooded organisms[2]. The investigation of the biological activity of saponins on insects was a good idea to be carried out although different studies have proved these actions of saponins[12–17].

Alfalfa saponins are triterpenoids composed of a C_{30} aglycone linked to one or more sugar groups as shown in Fig. 1 [18]. More than thirty-three different saponins containing one or more sugar chain units have been identified in alfalfa. Medicagenic acid, hederagenin, zanhic acid and soyasapogenols A and B are the main aglycones of alfalfa aerial parts[19,20]. Medicagenic acid is the first sapogenin synthesised in germinating seeds and the other sapogenins are formed from medicagenic acid[21]. The biosynthesis of

Abbreviations: Af, after flowering; Bf, before flowering; BuOH, *n*-butanol; MeOH, methanol; R_f, migration distance of band spots on TLC; Sdg, seedling.

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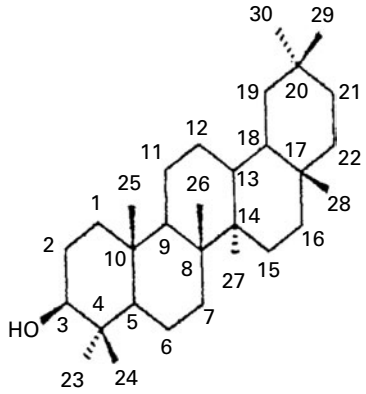

Fig. 1. Chemical skeleton structure of sapogenins.

sapogenins from [2-^{14}C]mevalonic acid was investigated in alfalfa and soyabean[20]. The addition of sugar moieties to sapogenins to form saponin glycosides varies during plant growth or development and results in changes in the overall saponin composition, which can have antibiotic or probiotic effects. Hydrolysis of a toxic or non-toxic saponin may also alter the biological properties of the compound. For example, Osbourn *et al.*[22] stated that the enzyme avenacosidase in oat activates the foliar oat saponins avenacosides A and B by the removal of C-26 glucose. This protein belongs to a family which also contains other plant enzymes involved in the activation of defensive secondary metabolites by the hydrolysis of glycosidic linkages, e.g. myrosinase (glucosinolates) and linamarinase (cyanogenic glycosides). Such compounds have been associated with a variety of biological activities, including allelopathy, poor digestibility in ruminants, enzyme activity inhibition, deterrence to foraging by insects and beneficial antifungal properties[23–25]. Activation resulting in alteration of metabolites is normally prevented by enzyme(s) and substrate(s) being separated in different cellular compartments, but is triggered by damage to tissues resulting from wounding or pathogen attack.

Saponins from alfalfa are also variable due to different factors. For example, when alfalfa foliages are damaged by an insect pest, a response will be induced in the plant and, consequently, more saponins will be either qualitatively or quantitatively induced[25,26]. Due to the presence of medicagenic acid glycosides which are found both in roots and in shoots, saponins in alfalfa are able to inhibit or reduce damage by insects, and soyasapogenol, and hederagenin glycosides found in the roots, which are rich in medicagenic acid derivatives, were markedly toxic to the flour beetle (*Tribolium castaneum*)[6]. Alfalfa saponins are also natural feeding barriers for phytophagous insects, and they were found to be toxic compounds to many insects[27]. Alfalfa saponins have been described as effective compounds interfering with aphid feeding behaviour[28]. An antibiosis mechanism of alfalfa saponins was the factor for this resistance[25,28]. Golawska extracted saponins 1, 2 and 3 from alfalfa with toxicity towards the pea aphid. Their toxicity potentials can be used as alfalfa resistance factors[27].

Variety, season, cultivar, environment[29–32], field drying[33], plant ensilage[34], age and plant part[35–38] are the other factors responsible for allelochemical variations in alfalfa and other plants. The amounts of saponins and also other secondary metabolites in plant tissues are variable and biologically affect insect pests such as the pea aphid (*Acyrthosiphon pisum* Harris)[28,39,40].

We were interested to investigate the biological role of saponins in the response of alfalfa (as a preferred host) to spotted alfalfa aphid (*Therioaphis maculata* Buckten) feeding, particularly in relation to changes in saponin composition during alfalfa development. This aphid, as an economically important pest of alfalfa, is also specific for different host plants such as onions, sainfoin, broad beans, clover, etc.[41]. In this research, we were also interested to know whether resistance to this pest could be produced by increasing the saponin levels or altering the saponin composition in the plant.

Materials and methods

Plant and insect materials

Alfalfa seeds (cultivar Euver) were sown in pots which were filled to the height of 10 cm with washed sea sand. The seeds were then covered with a 5 mm sea sand layer. The pots were placed in a greenhouse at the average temperature of 27°C under 16/8 h light–dark and irrigated every other day. Each pot was composed of two sections of a plastic drink bottle, with pores for air conditioning and preventing insects from escaping. For the evaluation of the effects of feeding on experimental alfalfa, for each treatment, four pots were used as four replicates. Plants were also transferred into bigger pots and moved outside the greenhouse for further experiments in developed growth stages.

Spotted alfalfa aphids were first reared on a present cultivation alfalfa cv. Hamedan. For more uniformity of the aphid colony, aphids were transferred to Blackman boxes containing alfalfa stem cuttings. Boxes were placed in a plastic tray containing water to the height of about 5 mm and then placed in the greenhouse at 27°C and 16/8 light–dark illumination. Produced neonates were used for bioassays at different stages of plant growth.

Tissues were also cut off from shoots at different growth stages, frozen in liquid N_2 and kept in a freezer at −20°C for saponin extraction.

Aphid bioassay

The response of the spotted alfalfa aphid to shoot tissues at different growth stages was investigated under greenhouse and field conditions in the Agriculture Faculty of Bu-Ali Sina University in Hamedan, Iran. In order to produce a colony of aphids, three adult aphids were removed from host plant leaves and placed on cutting stems in Blackman boxes. Five nymphs (1 d of age) produced by these adults

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were transferred from boxes and placed onto the leaves of the experimental plants in pots using a fine silk brush. Aphid survival, accumulative nymph production and the onset of nymph production were measured by daily observation on the experimental plants over a 2-week bioassay period. Treatments were analysed in the pattern of a completely randomised design with four replicates as Yazdi-Samadi *et al.*[42] suggested.

Extraction of crude saponins from alfalfa

Saponin extraction was carried out using the method described by Mazahery-Laghab & Gatehouse[16] and Mazahery-Laghab[17]. A quantity of 10 g of shoot (including leaves) and partly root tissues (in order to identify the sources of individual saponins) from alfalfa at different growth stages (seedling (Sdg) in greenhouse, after flowering (Af) and before flowering (Bf) outside the greenhouse in pots) was collected, frozen in liquid N_2, weighed out and placed in a cooled mortar. The tissues were finely ground using liquid N_2 to prevent any enzymatic degradation. The powdered tissue was then transferred to a conical flask and 50 ml of 80 % methanol (MeOH, 5 ml/g tissue) was added to the extracted saponins by stirring overnight at room temperature. The solution was filtered through a fine glass sinter, and the filtrate was evaporated under vacuum in a rotary evaporator at 40°C. Finally, 1·5 ml distilled water per g tissue was added to dissolve the residue. The resulting solution was stored and frozen at −20°C as crude saponin extract until required.

Chemical purification of saponins

A volume of 15 ml of crude saponin extract was transferred to a separating funnel and was mixed with 10 ml water-saturated *n*-butanol (BuOH). Two distinguishable phases, an upper BuOH layer and a lower aqueous layer, were formed after mixing and settling. Sometimes, an interface layer was also present depending on the sample being partitioned. The upper BuOH layer was first taken off and stored. The inter-phase layer was transferred into a centrifuge tube and centrifuged at >3600 rpm and the upper layer was added to the BuOH fraction. Aqueous supernatant was added to the lower H_2O layer and then the precipitate was removed. The combined aqueous layers were re-extracted twice with 10 ml BuOH as described above. The three BuOH layers were combined, evaporated in vacuum at 55°C, and then the residue was dissolved in MeOH. This method was scaled down for smaller amounts of material.

The saponin solution after extraction with BuOH was filtered through a glass micro-fibre filter CF/G. MeOH (5 ml) was used to wash the residue. The filtrate was mixed with five volumes of diethyl ether. The suspension, in a beaker covered by para film, was shaken and then left under a laboratory hood until a precipitate of saponins was formed, which was separated by centrifugation at >3600 rpm for 10 min. The precipitate was washed with diethyl ether until the diethyl ether wash solution was colourless after centrifugation. Residue pellets containing a pure mixture of saponins were dissolved in MeOH to use for TLC.

TLC

TLC was carried out on Alltech and Merck Silica Gel 60 F254 (Milano, Italy) 20 × 20 cm plates or as cut plates in 10 × 10 or 10 × 20 cm sizes. Pure saponin mixtures were analysed using TLC. After the centrifugation of the sample solution for 2 min, 10 μl of each sample was pipetted on TLC plate. The spotted samples were dried down with a hair dryer. TLC plates were then left inside a TLC tank lined with a filter paper and pre-equilibrated with solvent. The solvent system for saponin separation contained ethyl acetate–distilled water–acetic acid in the ratio 7:2:2 (by vol.). Plates were removed from the tank when the solvent front had reached approximately 1 cm from the top of the TLC plate. TLC plates were allowed to dry in air and then were sprayed with a reagent system, containing MeOH–acetic anhydride–H_2SO_4 in the ratio 10:1:1 (by vol.), freshly made. After spraying with this reagent, plates were transferred into an oven at 104°C for 15 min. Sprayed plates were observed under UV illumination (300 nm).

Identification of saponins using reference compounds

Either 1 mg of reference saponins or 10 mg of diethyl ether-precipitated purified saponin mixtures from alfalfa shoot and root tissues were dissolved in 1 ml of MeOH, micro-centrifuged, and after optimisation, 8·5 μl of solution of reference compounds (0·1 %) and 20 μl of purified saponin mixture (10 %) solutions were spotted on a glass TLC plate. As mentioned above, the plate was developed in a TLC tank containing the ethyl acetate solvent system. Different saponins in purified saponin mixture were compared with references (both under normal and UV light) after the staining of the TLC plate with saponin (H_2SO_4–acetic anhydride) reagent. Migration distances of band spots on TLC (R_f) of major spots were measured for comparison.

For the identification of alfalfa saponins, the total four saponins presented by Professor Dr Georges Massiot (Faculté de Pharmacie, URA CNRS 492, 51 Reims-Cedex, France) and Professor Dr W. A. Oleszek (Department of Biochemistry, Institute of Soil Science and Plant Cultivation, Pulawy, Poland) were used as reference compounds[19,26,43].

Results and discussion

Identification of alfalfa saponins using reference compounds

Saponin mixtures from alfalfa shoot and root tissues were spotted on glass TLC plate. A series of standard saponins

were obtained, and were also used to tentatively identify saponin spots on TLC, using similar R_f and similar spot colour after spraying with the acidic reagent as criteria for identification. Results are presented in Fig. 2. Purification and identification of saponins allowed specific components to be identified in comparison with reference tandards.

A monodesmoside contained soyasapogenol B as the aglycone; 3-*O*-α-L-rhamnopyranosyl (1 → 2)-β-D-galactopyranosyl (1 → 2)-β-D-glucuronopyranosyl soyasapogenol B (soyasaponin1)[43] gave a red-brown spot at R_f 0·33 (Fig. 2). A comparison between root and shoot saponin mixtures showed that this compound was purified in fractions eluted with 45–56 % *iso*-propanol and appears to be the major saponin present in both tissues, previously described as having R_f 0·43[16] (result not shown here).

A bisdesmoside 3-*O*-β-D-glucopyranosyl-28-*O*-(β-D-glucopyranosyl (1 → 4)-(α-L-rhamnopyranosyl (1 → 2)-α-L-arabinopyranosyl) medicagenic acid (medicoside J)[19] gave a dark olive-coloured spot at R_f 0·29, but did not appear to be present in the root or shoot saponin mixtures in the present work.

Another bisdesmosidic saponin 3-*O*-β-D-glucopyranosyl (1 → 2)-β-D-glucopyranosyl(-28-*O*-(β-D-xylopyranosyl (1 → 4)-(α-L-rhamnopyranosyl(1 → 2)-α-L-arabinopyranosyl) medicagenic acid[19] produced an olive green spot at R_f 0·19. An additional, fainter, purple spot at R_f 0·59 was also present in this sample, which could be identified as a 3-*O*-β-D-glucopyranosyl medicagenic acid in comparison with the other reference compounds. The additional spot had probably resulted from partial hydrolysis of the bisdesmoside. The shoot saponin mixture contained this compound as a major component; it was also present in a fraction eluted with 44 % *iso*-propanol[16].

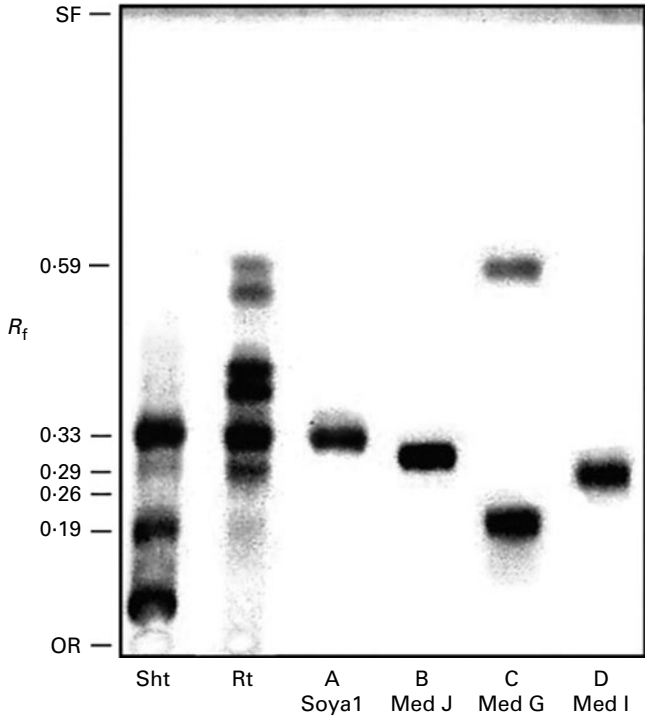

Fig. 2. Identification of saponin in alfalfa seedling roots (Rt) and shoots (Sht) by TLC using reference compounds. Migration distances of band spots R_f on TLC are given on the *Y*-axis of the figure. OR, origin of sample movement; SF, solvent front; Soya1, soyasaponin1; Med I, medicoside I; Med G, medicagenic acid glycoside; Med J, medicoside J.

The third bisdesmoside component, structurally identified as 3-*O*-(α-L-arabinopyranosyl (1 → 2)-β-D-glucopyranosyl (1 → 2)-α-L-arabinopyranosyl(-28-*O*-β-D-glucopyranosyl medicagenic acid (medicoside I)[19], was detected as a blue-grey spot at R_f 0·26. This compound appeared to be a minor component of the root saponin mixture, present in fractions which eluted with 52–56 % *iso*-propanol[16].

Although tentative identification of saponins can be made on the basis of R_f values on TLC, and values given in the literature, it is not valid to identify saponins on the basis of R_f values only, since mobility of components on TLC plates is subject to a high level of random variation between different TLC runs. However, the use of standard saponins of known structures, which can be run on the same TLC plate as unknowns, allows specific components to be identified. This identification is still not conclusive, since correspondence of spots on TLC does not prove identity, and single spots may be composed of more than a single saponin or aglycone, depending on the sample and the TLC system, but is sufficient as a working definition if other evidence is taken into account. In agreement with this conclusion, on the basis of TLC analysis, it was possible to identify major components of the crude saponin extracts in comparison with authentic compounds[44].

Changes of alfalfa saponins extracted from shoot tissues

Crude saponins were extracted from the shoot tissue of alfalfa in three different growth stages. Subsequently, the extracts were chemically purified and analysed by TLC to show changes in the saponin content that take place as the plant develops. The results of changes in alfalfa saponins during the development are shown in Fig. 3.

The saponin extract showed both quantitative and qualitative changes in components, as the alfalfa plant developed, with the intensity of spots increasing with plant age, suggesting that saponin content also increased. In addition, spots present in Sdg (e.g. a green-dark spot at R_f approximately 0·50) were not present in older tissues, showing that the chemical nature of the saponin fraction changed with plant age. The major changes were detected in the range of R_f 0·23–0·50 during the three growth stages (Fig. 3), and the colour and R_f values are listed in Table 1. It has also been stated that immature plants of a species have higher saponin contents than mature plants of the same species[2]. However, it seems to be dependent on the kind of species and also on the kind of saponin(s). A number of factors, such as physiological age, environmental and agronomic factors, have been shown to affect the saponin content of plants[2,45]. Reports reviewed

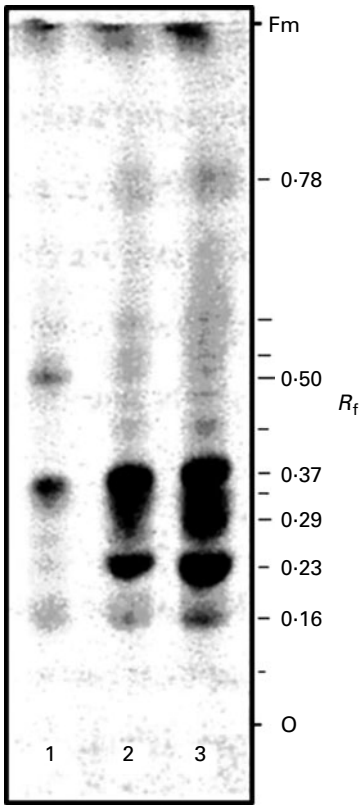

Fig. 3. TLC analysis of saponins extracted from alfalfa shoots in different growth stages. Migration distances of band spots on TLC (R_f) are given on the *Y*-axis of the figure. O, origin movement of solvent; Fm, final movement of solvent; 1, saponin extract from seedling tissues; 2, saponin extract from tissues before flowering stage; 3, saponin extract from tissues after flowering stage.

by Francis *et al.*[2] indicated that saponins increase on sprouting in some plants such as soyabean, alfalfa, mung beans and peas but decrease in others such as moth beans. Changes have also been observed in canavanine when alfalfa plants were developed. Saponin concentration rose to 8·7% in roots and 1·8% in shoots on the 8th day and slowly decreased to lower levels on the 24th day. No saponin in alfalfa seeds but 1% canavanine was reported[46]. Quantitative and qualitative differences in alfalfa shoot saponins have been reported by Bagheri *et al.*[47]. The average amount of crude extract of saponins from twenty-two cultivars was 0·81%. Saponin concentrations in different alfalfa varieties have also been reported and ranged from 0·8 to 2%[48,49]. In the present study, the concentration of saponins was not measured; however, saponin contents varied with alfalfa development. For example, Soya1 in both root and shoot tissues of alfalfa with an R_f of 0·29 (Fig. 4), also detected at R_f 0·50 on another TLC (Fig. 3), showed a relative decline in the amount as the plant developed, while a component of lower mobility at R_f 0·37 displayed an increase (Fig. 3). Some reports refer to the variations of secondary metabolites like saponins during plant development stages and some other reports refer to variations due to environmental factors[25–32,35–38,46].

As indicated in Fig. 4, an intense brown spot at R_f 0·26 was detected on TLC and identified as medicoside J. This compound had also been identified by Massiot *et al.*[19] as a bisdesmoside of medicagenic acid. Medicoside J did not appear to be present in the purified root or shoot saponin mixtures from Sdg tissues (Fig. 4) but appeared to produce a more intensive spot in mature alfalfa plants in Af (R_f between 0·37 and 0·50) as shown in Fig. 3.

Another saponin in both root and shoot tissues during the Sdg stage in alfalfa as medicoside I was visualised as an intensive spot at R_f 0·24 on TLC (Fig. 4) under UV light whereas this spot visualised at R_f 0·37 was given in Fig. 3. The colour of this compound was brown in shoot tissues under normal light (Table 1). Considering the reference component of F, this compound could be a kind of medicagenic acid which is identified as medicoside I, a bisdesmosidic compound (Fig. 4)[19]. The intensity of the spot of medicoside I on TLC increased as the plant developed.

Final accumulative spots at R_f 1·00 are hydrolysates of different components during extraction and purification. When the hydrolysis of components takes place, the products either disappear on silica gel or stop at higher R_f on the TLC plate depending on the kind of hydrolysed saponin or produced sapogenin (saponins with no sugar moiety). Disappearing components are probably sapogenins which are the hydrolysate of saponins. The latter components are probably different kinds of saponins on which one or two sugar moieties have been separated from aglycone moiety. So, it can be concluded that there are probably hydrolysing enzyme(s) in alfalfa tissues capable of producing new saponins (as resistance or non-resistance factors towards bio-stresses, i.e. toxic or non-toxic to insects or microbes). Mazahery-Laghab & Gatehouse[16] extracted a hydrolysing enzyme from alfalfa shoots and confirmed its responsibility for alfalfa saponin hydrolysis using TLC. Not only in the present study but also in previous studies, TLC was a potent technique to analyse alfalfa

Table 1. Migration distance of band spots on TLC (R_f) values and colours of spots detected in crude saponin extracted from the shoots of alfalfa during development under normal light on TLC

Number of spots (bands)	R_f values	Intensity of band spots			Colour
		Sdg	Bf	Af	
1	0·06	–	–	*	–
2	0·16	+	*	+*	Yellow-brown
3	0·23	+	+**	***	Olive
4	0·29	–	*	**	Green
5	0·37	*	***	***	Brown
6	0·43	–	+	*	–
7	0·47	–	–	+	–
8	0·50	+*	+	+	Green-dark
9	0·59	–	+	*	Yellow
10	0·75	–	*	+	Brown-yellow
11	0·78	–	+	*	Brown-dark
F12	1·00	+	+*	**	Brown

+, Compound with very weak intensity; *, low intensity; **, medium intensity; ***, high intensity; –, unclear; F12, final accumulative hydrolysed spots.

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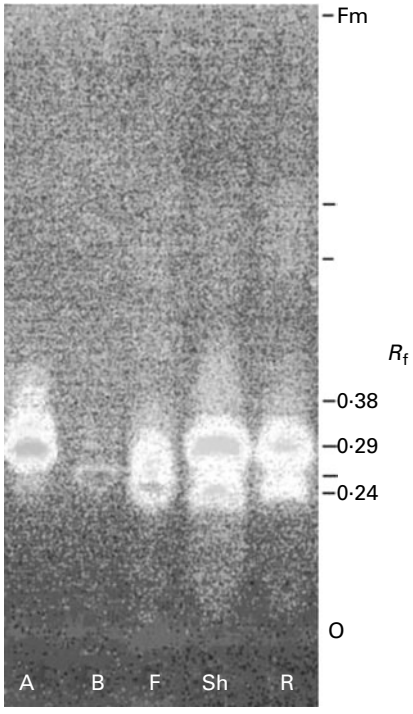

Fig. 4. TLC of pure saponin mixture from root (R) and shoot (Sh) tissues. Migration distances of band spots on TLC (R_f) are given on the *Y*-axis of the figure. A, B and F, presented standard saponins: soyasaponin1, medicoside J and medicoside I, respectively. O, origin movement of solvent; Fm, final movement of solvent.

saponins qualitatively[16,17] as also Oleszek[50] reported that TLC has a good potential to provide excellent qualitative information for determining plant saponins.

Analytical technique

Although many different procedures have been used for saponin analysis, such as foam production, haemolytic activity, inhibition of fungal growth, insecticidal or piscicidal activity, gravimetry, spectrophotometry, TLC, GC and HPLC[7,51], TLC was used as the principal analytical technique in the present study. While this technique has a number of drawbacks, in that it is semi-quantitative at best, and R_f values of specific components are not routinely reproducible among different TLC plates, these drawbacks are outweighed by its advantages.

Centrifugation was used in the preparation of samples to remove the insoluble materials that would distort the spot pattern. It was found that desalted samples gave a good chromatogram of spots, in agreement with Plummer[52], who stated that before chromatography, biological samples should be desalted using electrolysis or electro-dialysis. The presence of excess salts in the chromatography medium causes the spreading of spots on the plate and changes in their R_f values. To obtain reproducible results, it is also necessary to ensure a constant atmosphere in the solvent container. For this reason, during the development of the chromatogram, not only any exhausting of the evaporated solvent from the tank but also any importing of air from outside to the tank should be prevented. The tank should also be lined with filter paper, dipped in the solvent; this paper will keep the container saturated with the vapour of the solvent and will aid the ascent of the solvent front[53].

The stationary phase here is relatively polar, while the mobile phase is non-polar and acidic (and thus ensures that saponins containing carboxylic acid groups are uncharged). Saponins should thus separate on the basis of polarity; the less polar the saponin, the further it should migrate. For example, zanhic acid glycoside, a major triglycoside compound with a high polarity, appeared as the first spot at a low R_f with a high intensity after initial movement. High polarity of this compound makes it enter the water phase layer during water-saturated BuOH extraction. Previously, in shoot extracts, the 10 and 20 % MeOH fractions using column chromatography gave two green spots at R_f 0·14 and 0·20 on TLC[16]. It has been suggested that these spots may be zanhic acid tridesmosides; the presence of three glycosides makes this saponin relatively polar, leading to early elution from the reverse-phase column, and a low R_f value on TLC[54,55].

The coloured spots produced can be viewed under normal or UV light; the latter has the advantage that phenolic compounds give fluorescent spots, and thus can be distinguished from saponins. In the present study, by extraction with water-saturated BuOH, phenols were removed and disappeared on TLC.

Aphid bioassay

The survival and fecundity of the spotted alfalfa aphid that feeds on shoots of alfalfa cv. Euver at different plant developmental stages over a 2-week bioassay were measured.

Table 2. ANOVA for feeding effects from different growth stages on the biology of *Therioaphis maculata*

Traits	SOV	df	MS	Calculated *F*	CV%
Survivals	Growth stages	2	0·56	3·73 (NS)	8·51
	Error	9	0·15		
Accumulative nymph production	Growth stages	2	8990·08	11·41*	23·72
	Error	9	787·86	11·41*	23·72
Fecundity onset (d)	Growth stages	2	7·00	7·00**	11·76
	Error	9	1·00		

SOV, sources of variations; MS, mean of squares.
*,** Calculated *F* values were significantly different at 5 and 1 %, respectively.

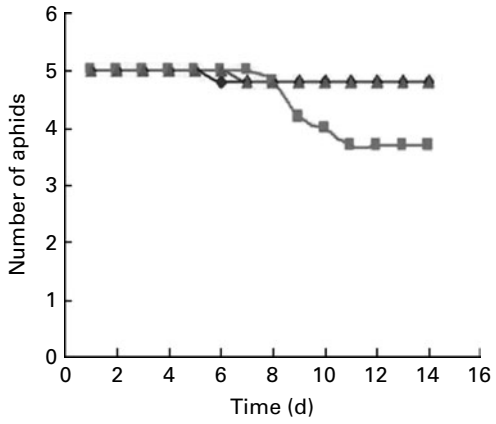

Fig. 5. Spotted alfalfa aphid survivals feeding on alfalfa plants at different growth stages. –◆–, Survivals on seedling stage; –■–, survivals on before flowering stage; –▲–, survivals on after flowering stage.

After 14 d, no significant difference in the survival of aphids among alfalfa plants of different ages was detected (Table 2). However, examination of the survival curves (Fig. 5) shows that survival remains high up to 8 d, but declines subsequently for aphids feeding on mature plants Bf, whereas survival on Sdg and mature plants Af remains high over the whole 14 d bioassay. However, there was no significant difference between the survivals of aphids in alfalfa growth stages (Table 2). There was no correlation between aphid survival and saponin contents as estimated by TLC, suggesting that this aphid is able to detoxify or is insensitive to the defensive compounds produced by alfalfa as a natural host. The aphid may be able to detoxify saponins by their hydrolysis to sapogenins. For example, it has been found that a hydrolysing avenacinase coded in *Gaeumanomyces graminis* removes β,1-2 and β,1-4 linked to D-glucose molecules from avenacin A-1 to give products with a lower toxicity to fungal growth[22,56]. However, sapogenins have been shown to be insecticidal in their own right[57]. The results of the investigation of the biological activity of medicagenic acid sodium salt and medicagenic acid glycosides from alfalfa Sdg on the growth of *Amaranthus*, *Lepidium* and tomato (*Lycopersicon*) cell growth showed that in contrast to medicagenic acid glycosides, medicagenic acid as sapogenin had stronger inhibition of plant and cell growth[46]. However, it is possible for some insects to have a potential to cleave one or more sugar moieties from saponins and change their biological activities[58].

Accumulative nymph production on shoot tissues of alfalfa was significantly different at the level of $<1\%$ probability in different growth stages (Table 2). The earliest onset of nymph production and the highest nymph production took place in mature plants at Bf stage (Fig. 6). Higher nutrient availability in phloem sap[59], production of new saponins (Fig. 3) and other metabolites such as flavonoids[39] and also lower concentration of Soya1 in Bf are probably some of the factors which have positive effects on the fertility of aphids. However, other than saponins, when plants contain other compounds, e.g. free sugars, phenolics and polar lipids[16], the effects could not be ascribable to any one compound. These is a combination of probiotic and antibiotic effects caused by different components[16,60] like saponins, which may express synergistic interactions in alfalfa, other plants and also insects[27]. A significant reduction in aphid fecundity at the level of 5% in Sdg and Af (Table 2 and Fig. 6) indicates that although saponin contents are comparably low in Sdg, the antibiotic effect of saponins are significantly too high, probably due to their quality and bio-activity. Therefore, an increase in the amounts of saponins in Af (compared to Bf) would result in an increase in the synergistic activity and the expression of antibiotic effects of saponins. In both the stages, the onset of nymph production was 2 d later than in Bf. Alfalfa saponins have also shown to reduce fertility of *Spodoptera littoralis*[61].

According to Fig. 6, although the fecundity of aphids feeding on alfalfa in Sdg and Af was similar and started on day 9, nymph number on Sdg was less than what was observed in other stage, i.e. Bf stage. This could be due to the high levels of saponins and the frequency of nutrients in the young leaves of Sdg tissues[16,59]. Young leaves of *Ilex opaca* Aiton with high level of nutrients were also unsuitable for the red mite *Oligonychus ilicis* McGregor due to the high levels of saponins[59]. However, the presence of Soya1 as the most effective compound in shoot tissues of alfalfa Sdg against the potato aphid (*Aulacorthum solani*), as Mazahery-Laghab & Gatehouse[16] reported in 1997, could be the main reason of the delay on onset of nymph production and a medium fecundity of spotted alfalfa aphids (Fig. 6). As shown in Fig. 5, the increased nymph production at the Bf stage corresponded to lower survival. Previous studies showed that Soya1 caused 60% mortality of potato aphids, delayed the onset of nymph production by 5 d and decreased aphid sizes by 29% compared with the control ones[16]. The onset of nymph production was significantly different at the level

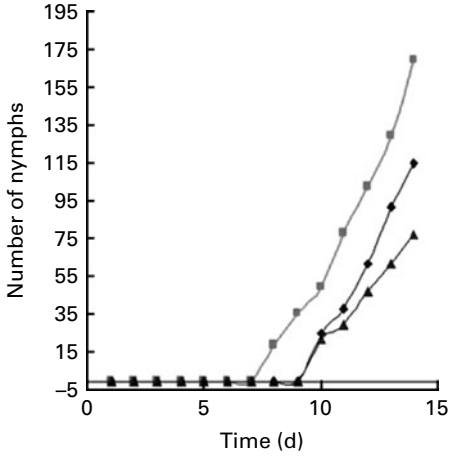

Fig. 6. Fecundity of spotted alfalfa aphid feeding on alfalfa plants at different growth stages. –▲–, After flowering stage nymph number; –■–, before flowering stage nymph number; –◆–, seedling stage nymph number.

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of <5% probability in three growth stages (Table 2). The fecundity of adults on alfalfa Sdg started on day 10, approximately 2 d later than aphids feeding on the shoots at developed ages in Bf. Again, this may reflect either a lower nutritional availability in Sdg or more toxicity of saponins during the Sdg stage as stated above. Exposure of the potato aphid to Soya1 from alfalfa Sdg caused a delay in nymph production (15 d compared with the control in which it occurred at day 12)[16].

In general, although saponins were quantitatively increased during plant development, less of a biological role was performed in the plant Bf in relation to both Sdg and Af stages. So, it can be concluded that the quantity of saponins can be overshadowed by their quality, e.g. presence of Soya1 in shoot tissues of alfalfa Sdg.

The data from these bioassays show that unlike the potato aphid, the spotted alfalfa aphid is able to tolerate the presence of saponins in its 'normal' host and that neither aphid survival nor development is significantly affected by the increased levels of saponins in older plants.

Conclusion

Data from bioassays carried out on plants at different developmental stages with different amounts of saponins present suggest that saponins are not effective as a defence against spotted alfalfa aphid attack, with neither survival nor fecundity showing a correlation with saponin content. This result contrasts with the insecticidal effects of alfalfa saponins on the potato aphid and shows that the spotted alfalfa aphid is adapted to the secondary defensive compounds present in its host plant. No significant difference in the survival of aphids, from neonate to adult, was observed, but two differences were found in the onset of nymph production and cumulative nymph production. The results also showed that the saponin composition in alfalfa changes with plant development and this, in turn, can affect the fecundity of even specific insect pests such as the spotted alfalfa aphid, concluding a possible biological role of alfalfa saponins.

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