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The Biological role of glycosides in alfalfa (*Medicago sativa* L.) as a resistance factor against alfalfa weevil (*Hypera postica* Gyll.)

Solhaddin Jamshidi-Golan[1], Sayyed Saeed Moosavi[1], Hamze Mazahery-Laghab[2], Majid Kazzazi[3], Hojatollah Mazahery-Laghab[1*]

[1]Department of Agronomy and Plant Breeding, Faculty of Agriculture, University of Bu-Ali Sina, Hamedan, Iran.
[2]Faculty of Medicine, Hamedan University of Medical Sciences, Hamedan, Iran.
[3]Department of Plant Pathology, Faculty of Agriculture, University of Bu-Ali Sina, Hamedan, Iran.
[*]Corresponding author, 0000-0002-1763-4689. Email: h.mazaherilaghab@basu.ac.ir.

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ABSTRACT

After the identification of resistant, semi-susceptible, and susceptible genotypes among 42 alfalfa (*Medicago sativa* L.) genotypes concerning farm traits, nine genotypes were selected. Saponins obtained as a result of homogenous suspension of alfalfa tissue under maximum alfalfa weevil (*Hypera postica* Gyll.) attack were extracted using methanol and were purified using water-saturated butanol. The resulting solution was immobilized using the active enzyme extracted from the midgut of the pest and the thermally deactivated enzyme. In addition to the isolation of secondary glycosidic metabolites extracted from alfalfa, the enzymatic hydrolysis activity of the pest in the glycosides of genotypes were monitored using thin layer chromatography. The results of the enzymatic digestion test using TLC indicated that other than glycosides1 epigenin, other compounds were digested in the solution. The flavonoidal band of epigenin glycoside1 in all five resistant genotypes, including Tak Buteh and Ranger, was high in density and nearly equal. While maintaining the stability of the molecular structure, this compound was isolated and revealed at Rf=0.45 on a TLC plate. Therefore, midgut enzymes of alfalfa weevil were not able to digest the above compound in alfalfa. In two susceptible genotypes of Poly Cross Shiraz and Mahali Neyshaburi, the saponin band of soysaponin1 at Rf=37 had the highest density. In natural field conditions and at the time of biological stress, the lowest concentrations of saponin, soysaponin 1, and the highest concentration of flavonoids, epigenin glycoside 1, had a significant biological role in resistance to pests, and reduced damage to the cultivars under study.

Key words: Alfalfa, Alfalfa weevil, Glycosides, Gut enzymes, Saponin hydrolysis.

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ABBREVIATIONS

DTT (Dithiothreitol (DTT) formula is $C_4H_{10}O_2S_2$), TLC (Thin Layer Chromatography), MeOH (Methanol the chemical formula is CH3OH), UV (Ultraviolet), UVP Chromato-VUE (UVP C-70G Chromato-Vue Cabinet from Analytik Jena), R_f value(for TLC) ($R_f = \frac{\text{distance traveled by sample}}{\text{distance traveled by TLC mobile phase solvent}}$), Ph (is a scale used to specify how acidic or basic (or alkaline) a water-based solution is), HPLC (High Performance Liquid Chromatography).

INTRODUCTION

The plants of the family *Leguminosae* have a variation in secondary metabolites not only as defense agents against pests, diseases and herbivores, but also as specific signaling compounds for insects attack. Some of these compounds, such as flavonoids and triterpenoids, have a wide range of distribution (Wink, 2013; Zaynab *et al.,* 2021). Saponin contents from legumes have been extensively studied. There is a big source of saponins with a biological activity (Jurzysta and waller, 1996; Oleszek, 2000). Changes in the contents of plant components have been frequently studied. The changes have been reported to depend on different factors like plant tissue, genotype, harvesting time, growth stage and bio-environment effects (Berrang *et al.,* 1974; Pecetti *et al.,* 2006). Saponins of alfalfa include complex glycoside triterpenoids, medicagenic acid, zanhic acid, hederagenin and soyasapogenol. These saponins possess a wide range of biological roles such as anti-nutritional, anti-fungal, anti-insect activities, toxicity to ruminants and hemolytic activities (Oleszek, 1996; Bialy *et al.,* 1999 and 2004). Saponins can act as protecting factors against bio-stresses. The results of Mazahery-Laghab *et al.* (2011) showed that saponin composition in alfalfa changes with plant development and this, in turn, can often negatively affect the development of specific insect pests such as spotted alfalfa aphid, suggesting a possible biological role of alfalfa saponins. Golawska *et al.* (2012) studied the effects of the quality and quantity of saponins in plant shoot on the development of pea aphid (*Acyrthosiphon pisum* Harris) in 4 cultivars of alfalfa. Agrell *et al.* (2004) studied the responses of alfalfa to phytophagous aphids and found that wounded plants on which *S. littoralis* larvae fed, proved 84% increase in saponin level. Additionally, the larvae avoided feeding on leaves which had been damaged for 5 to 7 days. Flavonoids as a group of secondary metabolites found in plants in the form of glycoside that could be attached to different groups of sugars like galactose, rhamnose, glucose and malonate glycoside, where the glycoside usually binds with R_2 and R_4 in flavonoid epigenin glycoside (Golawska *et al.,* 2012). Glycosides of flavonoids and free aglycones play a role in the processes of pathogenicity and symbionity with microorganisms. Epigenin glycosides are effective in plant-plant and insect-insect interactions (Nahrstedta, 1989). It has been supposed that flavonoids significantly affect the behavior and the function of insects (Simmonds, 2001; Lahtinen *et al.,* 2005). Many flavonoids have effect on the repulsion or suppression of insect attack (Lahtinen *et al.,* 2005; Simmonds, 2003). According to the research carried out by Kariyat *et al.* (2019) flavonoids of the sorghum plant are important defense compounds for the resistance of corn to leaf aphids *(Rhopalosiphum maidis).* (Hare, 2002). The flavonoids from aerial parts of alfalfa tissues are epigenin glycoside which have glucoronic acid in their sugar chain (Golawska *et al.,* 2012). The presences of digestive enzymes were reported to neutralize the toxicity of plant saponins in pathogen components as well (Bowyer *et al.,* 1995; Chandra and Chandra Pandey, 2008). Vatanparast and Hosseininave (2010) studied the digestion activity of α-amylase and pectinase on alfalfa weevil larvae and reported that α-amylase and pectinase enzymes extracted from insect midgut had the most digestion activity in the range of pH 5-6 at 35 °C, and at pH 5 at 40 °C. Chemical isolation mechanism of different components using chromatography technique depends on the solubility of compounds in a solution in the mobile phase and also the absorption and centralization of compounds in the mobile phase (Waksmundzka-Hajnos *et al.,* 2008). The study reported that, during the isolation of saponin, it is initially necessary to effectively extract the simple triterpenoid saponins, aglycones, and even mono-glycosides using a nonpolar solvent such as ethyl acetate. It was also expressed that the polar tridesmoside saponins such as zahnic acid glycoside adsorb and aggregate at low RF, whereas, bisdesmosides adsorb and aggregate in the middle sections and monodesmosides with the lowest polarity or no polarity adsorb at higher RF on TLC coated with silica gel. The toxicity of some secondary metabolites such as plant saponins depends on the glycosylation reaction. Some of the plants contain specific saponin hydrolyzing enzymes. These enzymes are able to eliminate one of the sugar chains from the whole saponin structure and induce a mono-desmoside compound which is more toxic than a bis-desmoside one (Osbourn, 1996; Minic and Jouanin, 2006) following a damage to plant tissues. The effect of saponins was also studied by Golawska *et al.* (2006).

They found that the alfalfa containing a high level of saponins proved to be better in development, survival and fertility of pea aphid compared to alfalfa with low level of saponins. Inaba *et al.* (2022) confirmed that some flavonoids effective in inhibiting Noppera-bo showed high larvicidal activity against *Aedes aegypti*. The present investigation tends to identify the alfalfa weevil resistance factor by extracting and identifying different types of glycosides from resistant, sensitive and semi-sensitive alfalfa genotypes selected based on field evaluation. The study of glycoside resistance against digestive enzyme activities of pest larvae, was also performed.

MATERIALS AND METHODS

Plant materials

After the analysis of the agronomic traits of the previous year and the identification of resistant, semi-resistant and susceptible genotypes among 42 alfalfa genotypes, nine genotypes were selected on the basis of field conditions (Table 1). Fifty grams of alfalfa shoot tissues were cut from each plot as a replication during pest attack and were wrapped in aluminum foil, then transferred to the lab immediately, frozen in liquid nitrogen and kept in a freezer at -20 °C until required.

Table 1. Alfalfa genotypes selected from germplasm population.

Genotype numeral.	Genotype name	Types of field resistance
1	Kerisary	Semi- susceptible
40	Hamedani	Semi- susceptible
28	Poly Cross Shiraz	Susceptible
34	Mahalie Neishaburi	Susceptible
9	Tak Buteh	Resistant
13	Feiz	Resistant
19	Ranger	Resistant
20	Bami	Resistant
32	Abadeh	Resistant

Extraction of digestive enzyme from the mid gut of alfalfa weevil

Weevil larvae were collected from a naturally infected field during pest attack in spring between April and May. The collected larvae were transferred to the lab in an appropriate plastic vials containing cut shoot tissues and kept temporarily in a fridge until required. Forty larvae, including third and fourth instar alfalfa weevil larvae were transferred from the plastic vials and immobilized by placing in an ice-cold beaker. The larvae were then transferred into a glass petri dish on ice and their heads and tails were removed using scissors, approximately 1mm from the ends of the body. Forceps were also used to facilitate the removal of the ends and removing the gut from the larval body. The removed gut was transferred to the tip of a Teflon pestle and placed into a glass homogenizer containing 10 mM DTT in distilled water (40 larvae in 1 ml buffer) and the guts were squeezed while the content was placed on ice. Further homogenization was carried out in the glass homogenizer. The amount of 50µl aliquots of homogenized gut extract containing digestive enzymes were pipetted into 1.5 ml Eppendorf tubes, frozen in liquid nitrogen and stored at -20 °C.

Thin Layer Chromatography

In order to use Thin Layer Chromatography (TLC), silica gel plates were purchased from Merk Company and used as fixed phase. Two solvent systems were also used in the mobile phase. The first solvent system was used to analyze crude saponins and it was composed of a 100 ml compound containing ethyl acetate, acetic acid, and distilled water in a volume ratio of 7:2:2. This solvent system was prepared under a fume hood (Mazahery-Laghab, 1997; Oleszek, 2000). The second solvent system was used to purify chemically pure saponins. It was composed of a 105 ml compound containing butanol, acetic acid, and distilled water in a volume ratio of 84:7:14. This solvent system was also prepared under fume hood (Golawska *et al.,* 2012).

Expression and visualization of saponins

TLC plates were sprayed with a reagent for saponins containing MeOH, acetic anhydride, and sulphuric acid in the ratio of 10:1:1 (v/v/v). After being sprayed with this reagent, plates were transferred to a drying oven at 104 °C for 15 min or 180 °C for 2.5 min. Sprayed plates were observed under normal light and under UV light at 300 nm, using a trans-illuminator (UVP Chromato-VUE). Each TLC plate was then photographed and R*f* values of the samples were measured and recorded. Selected TLC plates were photographed with an appropriate camera.

Chromatographic confirmation of digestive enzyme activity

In order to confirm the activity of extracted enzymes from insect gut using TLC, three samples from each genotype were studied: The first sample included 10µl pure saponin mixture. The second sample included 10µl pure saponin mixture plus 20 µl gut enzyme content. Later, the mixture was incubated at 25-35 °C and pH5.5-6.0 at room temperature for 12 h. The third sample was similar to the second except for the exposure of enzyme

content at 100 °C to a water bath for 5 min to inactivate the catalysis characteristic of the gut enzyme.

Estimation of the quantity and quality of saponins

For the determination of the relative quality of different saponins, the number of the visualized spots and their RF location on TLC plates were used. The results of Mazahery-Laghab (1997), Balestrazzi *et al.* (2011), Golawska *et al.* (2012) and Krishnamurthy *et al.* (2012) were used considering the unavailability of standard saponins (Table 2). The Rf values are equal to the distance migrated by the sample over the total distance traveled by the solvent. The below formula was used to calculate Rf values in the present study.

$$(1) \qquad R_f = \frac{\text{distance traveled by sample}}{\text{distance traveled by TLC mobile phase solvent}}$$

RESULTS AND DISCUSSION

In the present work, the ability of enzymatic digestion system of the pest on saponins extracted in butanol was studied using thin layer chromatography in 9 genotypes. As a result of this experiment, 7 band spots were visualized on the TLC plate. The bands were similar to the control spots (saponin extract without the presence of any extracted enzyme), (Figure 1). The results obtained from this experiment expressed the fact that an increase in environmental temperature would cause the deactivation of total enzyme extracted from the midgut of alfalfa weevil. No hydrolysis of saponins was detected after incubation of extracted saponins in DDT solution containing digestive enzymes at high temperature. This can be regarded as the natural characteristic of different biotypes of alfalfa weevil meaning that if there is no digestive enzyme for the hydrolysis and digestion of flavonoids or saponins in the midgut of pests, the insect cannot feed on or continue to feed on alfalfa. In the next step, the effects of alfalfa weevil of gut enzymes on the saponins of the same genotypes extracted in butanol were studied using thin layer chromatography (Figure 2). An interesting result was obtained. After the saponin samples were incubated with extracted enzymes for 12 h in pH 6, and 25-35 °C, six saponin bands were completely hydrolyzed and each sample produced a new spot at higher Rf on the TLC plate in comparison with the control. Only one band spot at Rf 0.45 was not hydrolyzed and produced no new spot after incubation with total digestive enzymes. This spot was related to the compound which was presumably a flavonoid as epigenin glycoside 1 and was not digested in a medium containing total enzymes from the pest

Table 2. The list of purified saponin band spots of alfalfa genotypes.

Number	Bond nam	R_f
1	zenhic acid tridesoside	0.10
2	Medicagenic acid glycoside tridesmoside	0.18
3	soyasapogenol glycoside	0.33
4	Soyasaponin I	0.37
5	apigenin glycoside 1	0.45
6	Medicagenic acid glycoside monodesmosid	0.58
7	apigenin glycoside 2	0.72

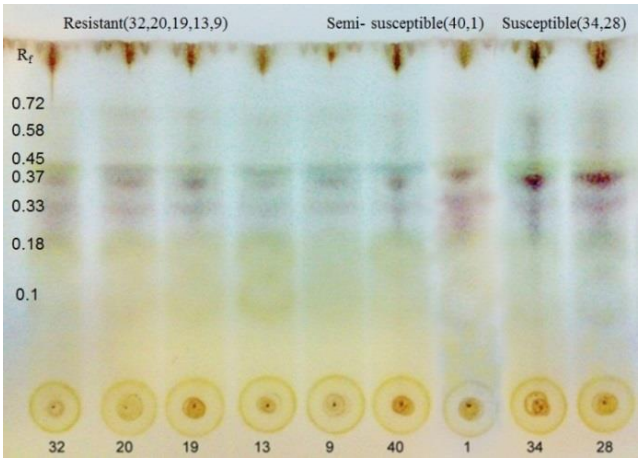

Figure 1. Profile of the band spots of purified saponins extracted from aerial parts of 9 alfalfa genotypes (mentioned in Table 1). Saponins were incubated with inactive enzymes during 12 h at room temperature.

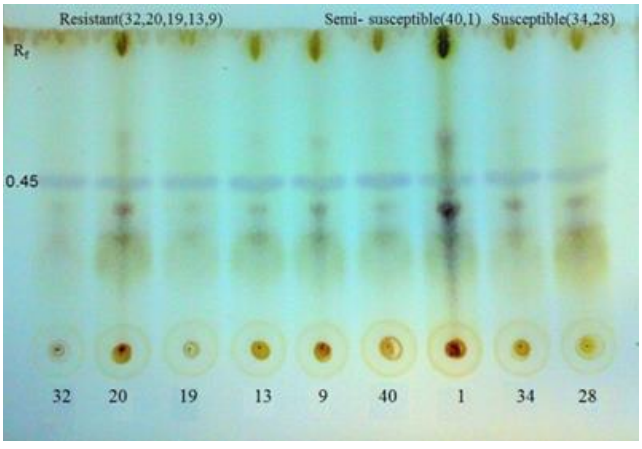

Figure 2. The bands of purified saponins extracted from 9 genotypes of alfalfa (mentioned in Table 1). Incubated with active enzymes for 12 h.

and it is comparable with the results of the following researches. Vatanparast and Hosseininave (2010) studied the digestion of the enzymes of α-amylase and pectinase extracted from alfalfa weevil. They measured the optimum pH and temperature appropriate for the activity of the enzymes in order to determine the profile of total plant flavonoids. Golawska *et al.* (2010) used High Performance Liquid Chromatography (HPLC). A high level of total flavonoids was reported, however, there was no significant difference between the concentration of this compound in the control and infected plants, and a negative correlation was found between the total concentration of epigenin glycoside 1 in alfalfa and the population frequency of pea aphid. It is concluded that considering the indigestibility of epigenin glycoside 1 by the midgut enzymes of alfalfa weevil, if this compound (flavonoid) is added with a high concentration to its daily artificial diet, it could pass the enzymatic barrier and be absorbed and preserved in the body of pests and finally cause biological problems. Molecular mechanism of biological effects of saponin on bio-membrane structure could be considered in this way that hydrophobic part of sapogenin combines with the sterol located in the cell plasma membrane and subsequently producing a complex of sapogenin-sterol, which causes the structural changes of membrane and the disordering of the midgut cell wall. Therefore, compound exchanges would not occur to cause the toxicity of animal cells (Armah *et al.,* 1999; Lin and Wang, 2010; Augustin *et al.,* 2011).

In order to compare saponin changes in resistant alfalfa genotypes, small samples were taken from the extract of the butanol phase, the extract incubated in inactivated enzymes and the extract incubated in active enzymes of the insect pest and spotted on silica plate. After the analysis and isolation of saponins, it was found that a flavonoid band spot related to epigenin glycoside 1 at RF=0.45 was similar in all 5 alfalfa genotypes. The concentration of this compound was also similar in all genotypes. This band stopped at the same Rf, i.e. 0.45, in the spotted samples on TLC plate, either incubated in active or in inactivate enzymes (Figure 3). The concentration of this compound was high in resistant genotypes in relation to other band spots on TLC and was not digested in enzyme assay when incubated with enzyme and had stability and preserved its molecular structure. In the final phase of the present research, the profile of butanol extract and incubated extract with active digestive enzyme from the midgut of the pest were compared in two resistant and two genotypes susceptible to alfalfa weevil (Figure 4). In the present work, flavonoid band spot of epigenin glycoside 1,

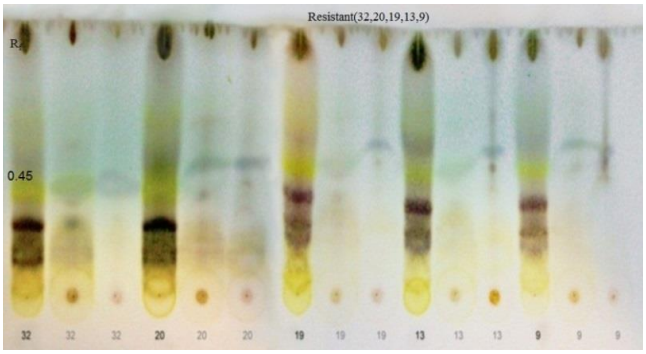

Figure 3. TLC profile of purified saponins extracted from 5 resistant genotypes in three conditions: pure extract, extract incubated with inactive enzymes and extract incubated with active enzymes from left to right, respectively, incubated for 12 h.

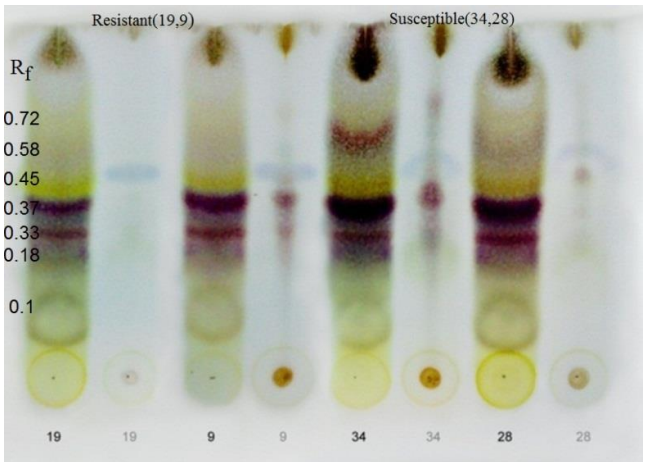

Figure 4. TLC profile of purified saponin extracts from two resistant (Ranger 19 and Tak Buteh 9) and two susceptible (Mahalie Neishburi 34 and Poly Cross Shiraz 28) genotypes incubated for 12 h without any enzyme and with enzyme from left to right, respectively.

had the highest band intensity at RF 0.45 in resistant genotypes TaK Buteh (9) and Ranger (19). The band related to Soyasaponin 1 at RF=0.37 had the highest intensity in genotypes Poly Cross Shiraz and Mahalie Neishaburi as susceptible genotypes which is similar to the results reported by Bede *et al.* (2006). They reported that, continuing pest feeding on aerial parts of the host plants, i.e. susceptible genotypes results in damages and injuries, and the amounts of medicagenic acid glycoside and the zanhic acid tridesmoside would increase significantly. They also found that effective concentrations of different saponins or non-saponin agents in resistant alfalfa genotypes have probably caused the expression of resistance reaction to the long time presence and feeding of the pest, so

the host plant has finally been protected from any further damage. Golawska *et al.* (2010). Reported that epigenin glycosides are known as protectors that protect plants against bio-stresses. These components usually play a major role in the physiology and development of plants, especially in the reaction of plants to other living organisms (Spaink, 1995). Both flavonoid glycosides and free aglycones are mediated in the reactions of pathogenicity and symbiosis with micro-organisms (Dixon *et al.,* 1994). Flavonoids are secondary metabolites that play an important role in plant growth by inducing resistance against biological and abiotic stresses (Shah and Smith, 2020). It seems that flavonoids affect the behavior and activity of insects (Simmonds, 2001; Lahtinen *et al.,* 2005). Some flavonoids could be effective in the excretion or restriction of insect attacks (Lahtinen *et al.,* 2005; Hare, 2002; Simmonds, 2003). These compounds have the ability to negatively affect the feeding behavior of insects (Schittco, 1999; Vanloon, 2002). However, other effects of plants special flavonoids on insects are still unknown. Flavonoid-rich parts of aqueous and methanolic extracts of *Glyceridia sepium* plants (belonging to the Leguminosae family), effectively kills mealybugs (Nukmal *et al.,* 2017). Therefore, it could be concluded that if an alfalfa genotype contains low levels of soyasaponin1 in contrast to the high levels of epigenin glycoside 1, it may lead to more resistance against insect pests under stress conditions.

CONCLUSIONS

The present work reported different concentrations of soyasaponin1 and epigenin glycoside 1 in individual alfalfa plants. These compounds had an effect on the performance of resistance against insect attack. So, it is suggested to investigate the effects of different concentrations of the above compounds simultaneously and non-simultaneously and to study the digestibility of secondary metabolites using digestive enzymes extracted from the midgut of insects.

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