Allelopathic activity of root saponins from alfalfa (*Medicago sativa* L.) on weeds and wheat

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Abstract. Bioassays were developed for allelopathic effects on dandelion (*Taraxacum vulgare*), coffee-weed (*Sesbania exaltata* L.), pigweed (*Amaranthus retroflexus* L.), barnyard grass (*Echinochloa crus-galli* L.) and cheat (*Bromus secalinus* L.) using pure alfalfa (*Medicago sativa* L., Cimarron cultivar) root saponins containing primarily medicagenic acid glycosides. Liquid secondary ion-mass spectrometry showed this cultivar contained 6 known saponins and several unknown saponins. The saponins were most effective allelopathically toward barnyard grass and cheat, less so for pigweed and coffeeweed with little effect on dandelions. The alfalfa root saponins were allelopathic toward wheat (*Triticum aestivum* L.), which was used as the control along with distilled water. The effect of alfalfa root saponins would have in the alfalfa field on the weeds tested remains unclear.

Key words: Allelopathy; *Amaranthus retroflexus*; Bioassay; *Bromus secalinus*; *Echinochloa crus-galli*; Liquid secondary ion-mass spectrometry; *Medicago sativa*; Saponins; *Sesbania exaltata*; *Taraxacum vulgare*; *Triticum aestivum*.

Introduction

Alfalfa produces allelopathic saponins, which may be a major cause of the reduction in yields of subsequent crops (Goplen and Webster, 1969; Gorski *et al.*, 1991; Guenzi *et al.*, 1964; Hall and Henderlong, 1984; Kehr *et al.*, 1983; Klein and Miller, 1980; Leshem and Levin, 1978; McElgunn and Hemrichs, 1970; Miller, 1983; Miller, 1992; Nielsen 1960; Oleszek and Jurzysta, 1987; Oleszek *et al.*, 1992a; Pedersen and Wang, 1971; Ream *et al.*, 1977; Wyman-Simpson *et al.*, 1991). The detailed chemical structures of many alfalfa saponins are known (Morris *et al.*, 1961; Gestetner *et al.*, 1971; Timbekova and Abubakirov, 1990, in press; Massiot *et*

al., 1988a, b, 1991; Oleszek *et al.*, 1990, 1992b; Levy *et al.*, 1989; Oleszek and Jurzysta, 1986; Morris and Hussey, 1965; Kitagawa *et al.*, 1988) with as many as 30-40 compounds with different properties of inhibition or stimulation being present in parts of the alfalfa plant. The plant parts differ greatly in the type and quantity of aglycones present, among which medicagenic acid glycosides have been shown to be the most biologically active saponins and are dominant in alfalfa roots (Gestetner, 1971; Oleszek and Jurzysta, 1986; Oleszek *et al.*, 1990, 1992). The aglycones identified from alfalfa saponins are primarily medicagenic acid (Table 1, I), and to a smaller extent hederagenin (Table 1, VI), lucernic acid (Table 1, IX), zhanic acid (Table 1, X), and soyasapogenol B (Table 1, XI), and to a much lesser extent soyasapogenol A, C, and E, and bayogenin (Djerassi *et al.*, 1988; Massiot *et al.*, 1988; Oleszek *et al.*, 1992b; Shany *et al.*, 1988).
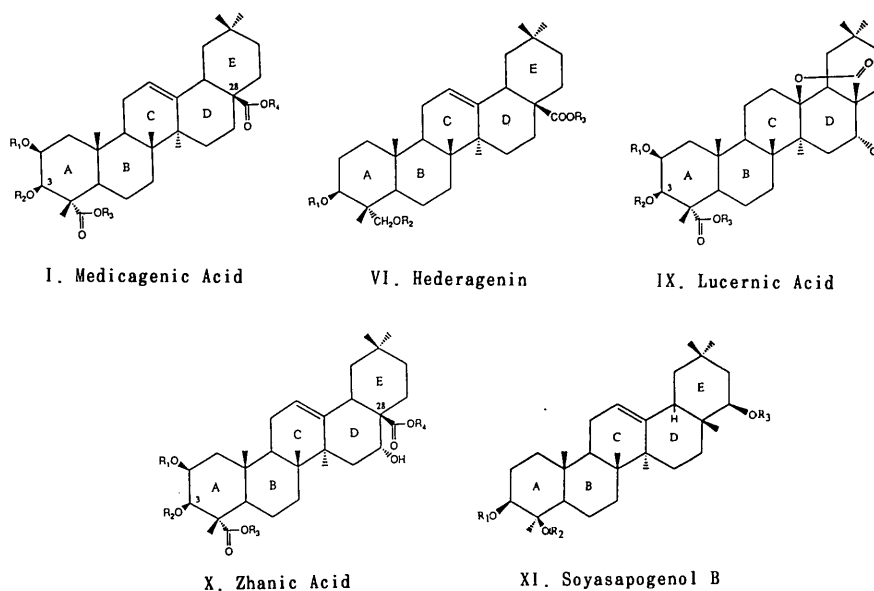
Reduced yields are commonly associated with old alfalfa stands and alfalfa planted directly after an old stand is plowed under. Yield from a five-year-old

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Table 1. *Chemical structures of some saponins and aglycones from alfalfa (**Medicago sativa** L., various cultivars) roots. Trivial nomenclature according to Timbekova et al.* (1990).

I. Medicagenic Acid   VI. Hederagenin   IX. Lucernic Acid

X. Zhanic Acid   XI. Soyasapogenol B

Trivial Name	Structure No.	R_1	R_2	R_3	R_4
Medicagenic acid	I	H	H	H	H
Medicoside A	II	H	β-D-Glu	H	H
Medicoside G	III	H	β-D-Glu	H	β-D-Glu
Medicoside H	IV	H	β-D-Glu	H	α-L-Rha $\xrightarrow{1\to2}$ α-L-Ara
Medicoside J	V	H	β-D-Glu	H	β-D-Xyl $\xrightarrow{1\to4}$ α-L-Rha $\xrightarrow{1\to2}$ α-L-Ara

		R_1	R_2	R_3
Hederagenin	VI	H	H	H
Medicoside C	VII	α-L-Ara $\xrightarrow{1\to2}$ β-D-Glu $\xrightarrow{1\to2}$ α-L-Ara	H	H
Medicoside I	VIII	α-L-Ara $\xrightarrow{1\to2}$ β-D-Glu $\xrightarrow{1\to2}$ α-L-Ara	H	β-D-Glu
Lucernic acid*	IX	H	H	H
Zhanic acid**	X	H	H	H
Soyasapogenol B	XI	H	H	H

*Lucernic acid; $R_4 = H$ on C_{27} and may be substituted.
**Zhanic acid; $R_4 = H$; $R_5 = H$ on C_{27} and may be substituted.

stand previously cropped in alfalfa has been as much as 32% lower than from a stand not previously so cropped (Kehr *et al.,* 1983). Even if soil moisture, fertility, and pest problems are corrected and alfalfa is reseeded, attempts to increase production from old alfalfa stands are frequently unsuccessful. Reduced yields of other

crops are often seen when these are grown in rotation with alfalfa, such as wheat (*Triticum aestivum* L.), corn (*Zea mays* L.), soybeans (*Glycine max* L.), and sorghum (*Sorghum bicolor* L.).

Mishutin and Naumova (1955) first suggested that saponins, leached into the soil from alfalfa roots for 3-4 years, are allelopathic agents, causing a decrease in the yield of cotton (*Gossypium arboreum* L.) planted directly after alfalfa, especially older stands of alfalfa; however, most grain crops were not sensitive to this level of saponins. Jurzysta (1970) reported that *Medicago lupulina* seed saponins (purified) reduced the growth of wheat (also oats, barley, and rye) seedlings at concentrations of 0.1-0.5%; however, the same concentrations of *Medicago media* seed saponins had no influence. Jurzysta (1973) reported on the chemical

structures of *M. lupulina* and *M. media* seed saponins, and found that the former contains medicagenic acid glycosides and soyasaponol glycosides whereas the latter contained only soyasapogenol glycosides; this explains the difference in inhibition toward wheat seedlings of the two varieties of alfalfa. Oleszek and Jurzysta (1986) reported that medicagenic acid glycosides isolated from *Medicago media* roots were much more biologically active than glycosides of hederagenin or soyasapogenol B. Miller *et al.* (1988) implicated medicarpin, a phenolic phytoalexin, and a germination and growth inhibitor. Dornbos *et al.* (1990) reported

that medicarpin causes only delayed autotoxicity of alfalfa. These authors also isolated medicarpin from alfalfa soil and found that alfalfa plants metabolize it. Read and Jensen (1989) showed that water-soluble or methanol-soluble compounds from alfalfa soil caused decreased growth of barley (*Hordeum vulgare* L.)

tions only a stimulating activity was observed. These results indicate that the differences in allelochemical activity of the saponins may be due to structural variations (Oleszek *et al.*, 1990, 1992 a,b; Tarikov *et al.*, 1988; Timbekova and Abubakirov, personal communication; Waller, 1989a, b).

The purpose of this research was to determine, using bioassays, if purified alfalfa root saponins were allelopathic toward coffeeweed, dandelion, pigweed, cheat, and barnyard grass. Distilled water and wheat were used as controls.

Materials and Methods

Alfalfa Plant Material

Seeds and roots of alfalfa (*Medicago sativa* L. cultivar Cimarron) collected from 1985 to 1988 were obtained from the Department of Agronomy, Oklahoma State University, Stillwater, OK 74078, USA. The top 10 cm of the tap root and the lateral roots of the plants were taken for isolation of the saponins. Roots of plants exhibiting signs of fungal, bacterial, or nematode damage were not collected. Excess soil was removed and the roots were dried 30 h at 60°C in a circulating air oven. Residual soil was removed from the dried roots with a brush. Roots were not originally washed with water to avoid saponin loss.

Alfalfa Roots Extraction, and Purification Procedure

The dried roots were ground to pass a 0.6-mm screen in a Wiley mill (Arthur H. Thomas Co., Philadelphia, PA). A modification of the procedure according to Jurzysta *et al.* (1984) was used. Three hundred grams of the ground alfalfa roots were extracted with chloro-

to obtain the completely precipitated saponins. The saponins were filtered out on a sintered glass (G-3) filter and washed with benzene until the filtrate was free of cholesterol as indicated by negative reaction with the Lieberman-Burchard reagent. The saponins were washed once more with 100 ml of ethyl ether and dried at 70°C. The saponins were collected as a mixture of solid crystalline compounds, slightly cream in color.

Chemical Reagents and Test Plants

All solvents used were Baker Resi-Analyzed grade (J. T. Baker Chemical Company, Phillipsburg, New Jersey, USA.) Wheat (*Triticum aestivum* cv. Pioneer 2157) seeds used were obtained in March 1985, and had been stored at 4°C. Cheat (*Bromus secalinus*) seeds were collected locally in 1987. Other weed seeds were obtained from the Valley Seed Service, Fresno, CA, USA and stored at 4°C.

Mass Spectrometry Analysis of Saponins-by Liquid Secondary Ion Mass Spectrometry (LSIMS)

The LSIMS (Liquid Secondary Ion Mass Spectrometry) mass spectrometer used was a VG-70-250 S (VG Tritech, Manchester, United Kingdom) equipped with a Cs+ gun at 35 KV for the ionization. The saponins were introduced in three methods: a)

solid form, b) dissolved in CH_3OH, and c) dissolved in water. Glycerol, thiogycerol, and 50:50 glycerol:thioglycerol were used as matrices.

Wheat and Weed Bioassays

Bioassay experiments were designed to measure the early growth of wheat and weeds treated with purified alfalfa root saponins as compared to a distilled water control. Purified alfalfa root saponins were dissolved in distilled water at concentrations of 0.001 (10 ppm), 0.010 (100 ppm), 0.100 (1000 ppm) and 0.500 (5000 ppm) percent.

Approximately 75 wheat or weed seeds were placed on a 9.0-cm disk of Whatman No. 1 qualitative filter paper in the lid of an inverted 100×15 mm plastic Petri dish. The filter paper was soaked with 3 mL of distilled water and the seeds were incubated on the laboratory bench (20-30°C, 10 h light: 14 h dark) for 48 h. Two 1 mL additions of distilled water were made during this time to each dish to keep the filter paper soaked.

After the 48-h incubation, 100×15 mm glass Petri dishes were prepared for the bioassay. Each dish was lined with a 9.0-cm disk of Whatman No. 1 filter paper and 2 mL of the test solution was applied.

Ten seedlings of uniform size (root lengths within 3

Table 2. *Partial positive LSIMS analysis of known and unknown saponins from alfalfa (**Medicago sativa L.. Cimarron** cultivar) roots*

Trivial Name	A: *Known Compounds*: Oleszek *et al.* (1990) Technical Name	LSIMS Molecular Weights with some characteristic peaks**
Medicoside A	3-β-D-Glu Medicagenic acid (MA)*	665, 503, 457, <u>443</u>
Medicoside G	3-β-D-Glu, 28-β-D-Glu MA	849, 665, 503, <u>457</u>
Medicoside H	3-β-D-Glu, 28-α-L-Rha $\xrightarrow{1\to2}$ α-L-Ara MA	1097, 1075, 943, 665, 503, <u>457</u>
	3-β-D-Glu, 28-β-D-Xyl $\xrightarrow{1\to4}$ α-L-Rha $\xrightarrow{1\to2}$ α-L-Ara MA	1097, 1075, 943, 665, 503, <u>457</u>
	3-α-L-Ara (1→2)-β-D-Glu (1→2) α-L-Ara Hederagenin	789, <u>767</u>, 605

mm) were arranged in a radial pattern with the micropylar end toward the center on the premoistened filter paper. Each treatment was replicated four times. Seedlings were incubated in the dark at 20°C for 72 h.

Lengths of central roots and coleoptiles of each seedling were measured. Any seedling showing signs of visual fungal or bacterial contamination was discarded. Any changes in the appearance of the seedlings, such as root tip browning and necrosis, were discarded. Means per dish and per treatment were calculated and Student t-test was used in the data analysis (Steel and Torrie, 1985).

Results

Chemical Composition of Alfalfa Root Saponins (Cimarron cultivar)

The same Cimarron cultivar was used as a standard in the paper by Wyman-Simpson *et al.* (1991) and was found to contain a mixture of saponins as well as

between the listed known compounds and the spectra reported in Fig. 2. As can be seen from Fig. 2, none of the fragmentation peaks are shown in these spectra; an exception is at m/z 455 at which the base peak of the Medicoside H occurred. This compund was in the Polish aerial (Oleszek *et al.*, 1992b), and the Russian roots of the alfalfa plant (Timbekova and Abubakirov, 1986). The unknown saponins which were first shown present by thin-layer chromatography by Wyman-Simpson *et al.* (1991) are quite reasonable ones from the four aglycones reported. It is possible that combinations of techniques, such as linked scan (B/E) MS/MS, successive HPLC, mild hydrolysis, and ^{13}C NMR can identify some of these unknown saponins in a complicated mixture.

Choice of Bioassay Method

Carefully selected wheat seeds have a germination frequency of nearly 99%, but weed seeds have a much lower frequency, less than 60%, as is typical of the

the aglycones by thin-layer chromatography; the aglycones were identified as medicagenic acid at R_f 0.47 ± 0.05, hederagenin at R_f 0.62 ± 0.04, luzernic acid at R_f 0.40 ± 0.05 (Massiot, G. personal communication), and soyasapogenol B at R_f 0.57 ± 0.05. The LSIMS (Table 2) showed six compounds that were identified by Timbekova and Abubakirov (1986, in press), (Timberkova *et al.*, 1990) Massiot *et al.* (1988, 1991), and Oleszek *et al.* (1990, 1992 a, b) as occurring in the French, Polish, and

seeds of many weedy species. Weed seeds presumably have a much higher degree of genetic variability than wheat seeds, so the length of time required for germination differs considerably. The incidence of bacterial and fungal contamination following incubation is greater in weeds than in wheat.

Leather and Einhellig (1985) compared the sensitivity of various types of bioassay designs. They found that bioassays involving pregerminated sorghum (Sor-

*Comparison of Growth of Weed and Wheat*

Figures 2-7 show average wheat and weed growths in the presence of varying concentrations of pure alfalfa root saponins from the Cimarron cultivar. Standard deviations of the means were fairly small, indicating nearly uniform growth response of the wheat and weed seedlings to a certain type of treatment. The effect of pure alfalfa root saponins on dandelions (Fig. 3) was insignificant when compared to wheat (Fig. 2). Coffeeweed (Fig. 4) showed small (20-40%) stimulatory responses to low concentrations of 10 and 100 ppm of the saponins; however, at 1000 and 5000 ppm saponins strongly inhibited the growth of coffeeweed roots

8102118282   x1   Bgd=1   2-FEB-92   18:43+8:88:58   FB+
I=3.9v   TIC=175488888
Medicago sativa, Cimarron cultivar, root saponins, pure, crystalline
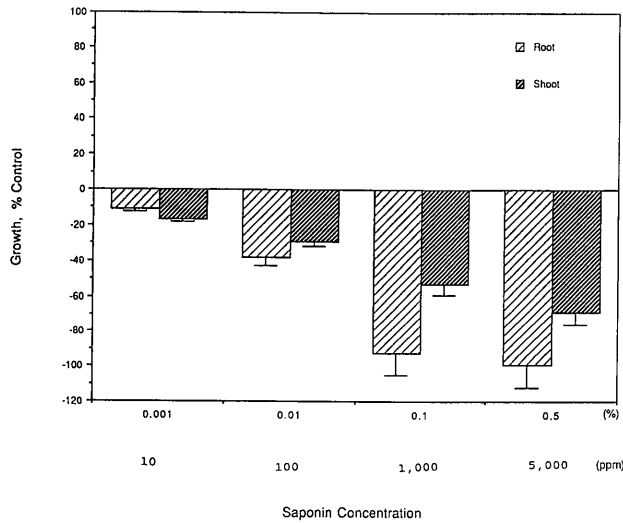
Root
Shoot
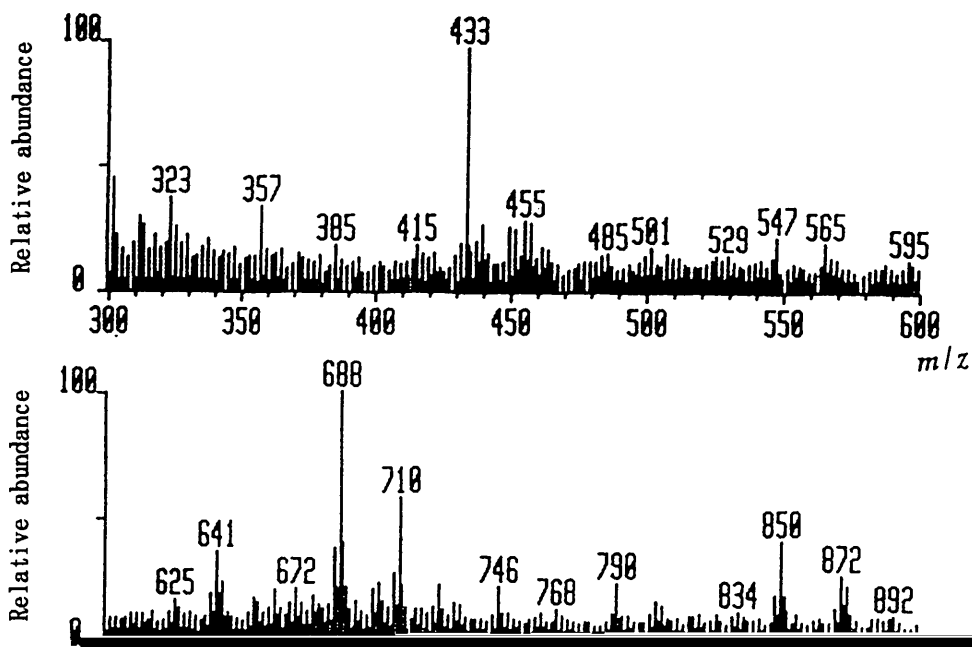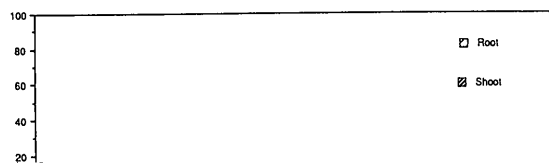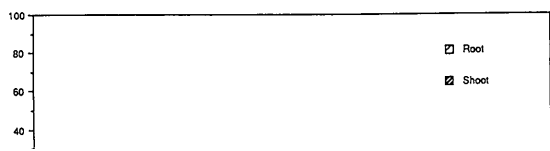
Root
Shoot

Fig. 6. Growth of barnyard grass (*Echinochloa Crus-galli* L.) as a percentage of the distilled water control in the presence of different concentration of crystalline saponins from alfalfa roots (Cimarron cultivar).
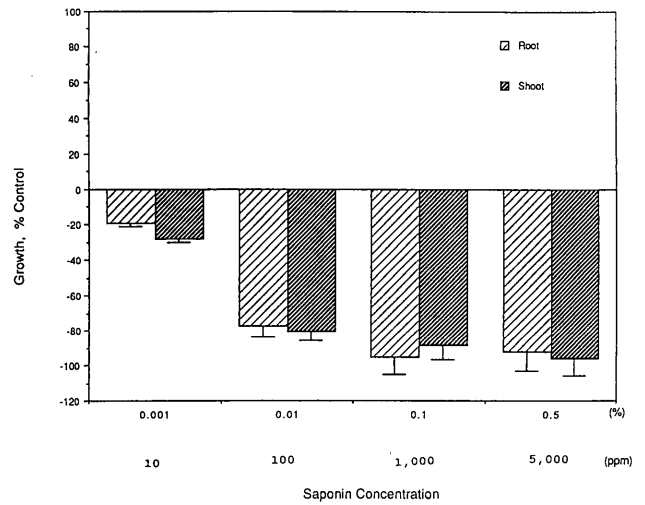

Fig. 7. Growth of cheat (*Bromus secalinus* L.) as a percentage of the distilled water control in the presence of different concentration of crystalline saponins from alfalfa roots (Cimarron cultivar).

concentrations of 1000 and 5000 ppm only small differences (10-15%) in growth were observed.

Discussion

The marked variability in the phytotoxicity of the water extracts to wheat and cheat seedling growth with time was seen also by Guenzi *et al.* (1964), and Pedersen (1975). Guenzi *et al.* found that extracts of alfalfa forage at the bud stage highly inhibited corn seedling root growth, but extracts from alfalfa harvested 25 days after full bloom were least inhibitory. The allelopathic action of alfalfa meal in the control of root rot caused by *Phytophthora cinnamoni* (Zentmeyer, 1963) was found to be due to the saponin content (Zentmeyer and Thompson, 1967). *Trichoderma viride* as well as other fungi were unusually sensitive to alfalfa saponins, and this property led to the development of a bioassay (Zimmer *et al.*, 1967)., Modifications of this bioassay are still used for alfalfa saponins evaluation (Jurzysta, 1979, Wyman-Simpson *et al.*, 1991); however, its use has been found less desirable, leaving the saponin filed (Oleszek *et al.*, 1992b) without a suitable bioassay method. Compound II (Table 1) isolated from alfalfa roots demonstrates high activity against *T. viride*, and some important plant pathogens: *Sclerotium rolfsii, Rhizopus mucco, Aspergillus niger, Phytophthora cinnamoni, Fusarium oxysporum*, f. sp. *lycopersici* (Levy *et al.*, 1986). Medicoside U (structure II in Table 1) was

also toxic to ten medically important yeasts (*Candida* spp., *Torulopsis* spp., and *Geotrichum candidum*) (Polachek *et al.*, 1986); stability, and strong antifungal activity suggest that it might have a role in the treatment of mycotic infections.

Six alfalfa root glycosides were tested for antimicrobial activity as well as their inhibitory effect on the germination of cabbage (*Brassica oleracea* L.) seeds by Timbekova, Abubakirov, Boguslawsky and Burceve (private communication). Medicosides were tested as shown in Table 1. Compound II showed inhibition of germination, whereas VII showed only a slight inhibitory effect but stimulated the growth of some microorganisms. A direct correlation between the polarity of medicagenic acid bisdesmosides, III, IV, and V was observed; these polar glycosides inhibited cabbage seed germination more than glycosides with similar carbohydrate chains. The activity of the terpenoid glycosides in inhibiting the microorganisms and the fungi growth increased with the polarity. Similiar degrees of performance were noted for the polarity of the hederagenin glycosides VII and VIII. The phytopathogenic microorganisms which were inhibited by six triterpenoids (II -VIII) were: *Agroacterium tumefaciens, Corynebacterium michiganense, Pseudomonas lachrimans,* and to a lesser extent *Corynebacterium insidiosum. Xanthomonas campestris* was inhibited by only saponins from the aerial parts of the plant.

The antifungal, antimicrobial, and allelopathic

activity of alfalfa saponins depends to a large extent upon the medicagenic acid glycosides and to a lesser extent on hederagenin and zhanic acid glycosides (Oleszek *et al.,* 1992b). It was first suggested by Oleszek *et al.* (1988a) that the antifungal activity depended upon free hydroxyl and carboxylic acid groups of medicagenic acid, and that the maximum activity was associated with free R_1, R_2 (OH) and R_3, R_4 (COOH) groups (Table 1). We believe it is proper and reasonable to extend these findings to explain the allelopathic and antimicrobial activity as well.

Wyman-Simpson *et al.* (1991) suggested that the metabolic pool of alfalfa root saponins (Cimarron cultivar plus six other cultivars selected for their dormancy) was subject to small changes with time and location depending upon where the alfalfa was grown in Oklahoma. The same sample of alfalfa root saponins (Cimarron cultivar) was used in this research. The predominant evidence now indicated that cultivars of *Medicago sativa* L. grown in different parts of the world (Gorski *et al.,* 1991; Massiot *et al.,* 1988; Oleszek *et al.,* 1990; and Wyman-Simpson *et al.,* 1991) do possess slight differences in the composition of root saponins, but these differences are not enough to prevent them from having the same relative allelopathic activities. This research confirms and extends the finding that extracts of alfalfa roots can serve as allelopathic agents that are effective against dandelion, coffeeweed, pigweed, barnyard grass and cheat. They are also allelopathic against wheat. Clearly more evidence is needed before alfalfa saponins could be used as an effective weed control.

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苜蓿根中皂苷對雜草及小麥之相剋作用活性

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應用生物分析法研究純苜蓿 (*Medicago sativa* L., Cimarron cultivar) 根中包括初級苜蓿酸配醣體 (medicagenic acid glycosides) 皂苷對蒲公英 (*Taraxacum vulgare*)，田菁 (*Sesbania exaltata* L.)，莧 (*Amaranthus retroflexus* L.)，小稗 (*Echinochloa crus-galli* L.) 和雀麥 (*Bromus secalinus* L.) 相剋作用效應。高速原子撞擊質譜顯示此栽培種包括 6 種已知的皂苷及 25 種未知的皂苷。其對小稗及雀麥的相剋作用最強，莧及田菁次之，對蒲公英極弱。蒸餾水作為對照時，苜蓿根中皂苷對小麥 (*Triticum aestivum* L.) 有相剋作用。而此皂苷在苜蓿田中，對於所試驗雜草的作用仍未知。