

Effects of Alfalfa Saponins on *In Vitro* Physiological Activity of Soil and Rhizosphere Bacteria

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SUMMARY. *In vitro* effects of purified alfalfa (*Medicago sativa* L.) saponins (soyasaponin I, medicagenic acid [MA-3,28Na], MA-3glu, 28Na, and MA-3,28glu) on rhizosphere bacteria cell suspensions were investigated. Triphenyltetrazolium chloride (TTC)-dehydrogenase and fluorescein diacetate (FDA) hydrolytic activities were strongly inhibited (90-95%) by MA-3,28Na and MA-3glu,28Na in *Bacillus thuringiensis* strains (HD-2 and UZ404). In *Curtobacterium flaccumfaciens* (JM1011), TTC and FDA activities were reduced only by MA-3,28Na, while little or no effects were observed on the gram-negative strains *Pseudomonas fluorescens* (RA-2) and *Agrobacterium tumefaciens* (A-136). Soyasaponin I decreased FDA hydrolysis in HD-2 and JM1011, but increased FDA activity by 2-fold in RA-2. MA-3glu,28Na increased protein exudation or leakage by 5 to 10-fold in all strains, and MA increased leakage in all strains except RA-2. Soyasaponin in-

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creased protein leakage about 2-fold in JM1011, RA-2 and A-136, but reduced extracellular protein in HD-2 and UZ404. Results suggest that MA-3glu,28Na can deleteriously affect rhizobacteria, and in most cases, the aglycone (MA-3,28Na) is as biologically active as the saponin MA-3glu,28Na. [Article copies available for a fee from The Haworth Document Delivery Service: 1-800-342-9678. E-mail address: <getinfo@haworthpressinc.com> Website: <<http://www.HaworthPress.com>> © 2001 by The Haworth Press, Inc. All rights reserved.]

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INTRODUCTION

Saponins are comprised of plant glycosides which contain a polar moiety; i.e., water-soluble sugar(s) attached to a lipophilic moiety of steroid or triterpenoid nature. These compounds are found in roots, shoots, seeds, and flowers of many plant species. They are of agronomic interest because of allelopathic interference with plant growth (Oleszek and Jurzysta, 1987; Waller, Jurzysta, and Thorne, 1995). The ecological significance of plant saponins is of interest and has recently been reviewed (Oleszek, Hoagland, and Zablotowicz, 1999). Various saponins exhibit antifungal activity, including toxicity to plant pathogenic fungi. Saponins can influence the growth of soil microflora, and the fungus *Trichoderma* spp. was found to be extremely sensitive to low saponin concentrations (Mishustin and Naumova, 1955; Zimmer, Pedersen, and McGuire, 1967). The effects of β -escin and several other saponins on the growth and activity of several genera of rhizosphere bacteria have also been reported (Zablotowicz, Hoagland, and Wagner, 1996). A wide range of sensitivity to various saponins was observed among bacterial species. Certain bacteria, especially those of the Rhizobiaceae (*Bradyrhizobium*, *Agrobacterium* and *Rhizobium* species) were most susceptible to growth inhibition, while *Bacillus* sp. were most prone to a loss of metabolic activity.

Alfalfa (*Medicago sativa* L.) produces allelopathic saponins which have been implicated as a cause of yield reductions in subsequent crops. Chemical structures of many of these compounds have been determined, and differential biological activity has been associated with differing structures (Waller, Jurzysta, and Thorne, 1995). Alfalfa plant parts differ greatly in the type and quantity of aglycones and saponins present in these tissues (Nowacka and Oleszek, 1994). Among these, medicagenic acid glycosides have been shown to be the most biologically active saponins. They are dominant in alfalfa roots

(Oleszek, Hoagland, and Zablotowicz, 1999). Other alfalfa saponins such as soyasaponin I occur in several genera and species of legumes. The effects of saponins from alfalfa and other plants have been studied in both phytopathogenic and nonpathogenic fungi and found to be inhibitory to a variety of fungal species (Zimmer, Pedersen, and McGuire, 1967; Levy et al., 1989, Oleszek et al., 1990, Oleszek, 1993). Certain alfalfa saponins have also been shown to be inhibitory to some phytopathogenic bacteria (Timbekova, Isaev, and Abubakirov, 1996). Although, alfalfa saponins are allelopathic to plants and inhibitory to certain fungi, their effects on bacteria have not been as widely studied and are poorly understood. Thus, our objectives of these experiments were to critically assess the effects of highly purified alfalfa saponins on a variety of rhizosphere bacteria using *in vitro* parameters that have been shown to be useful indicators of saponin toxicity.

MATERIALS AND METHODS

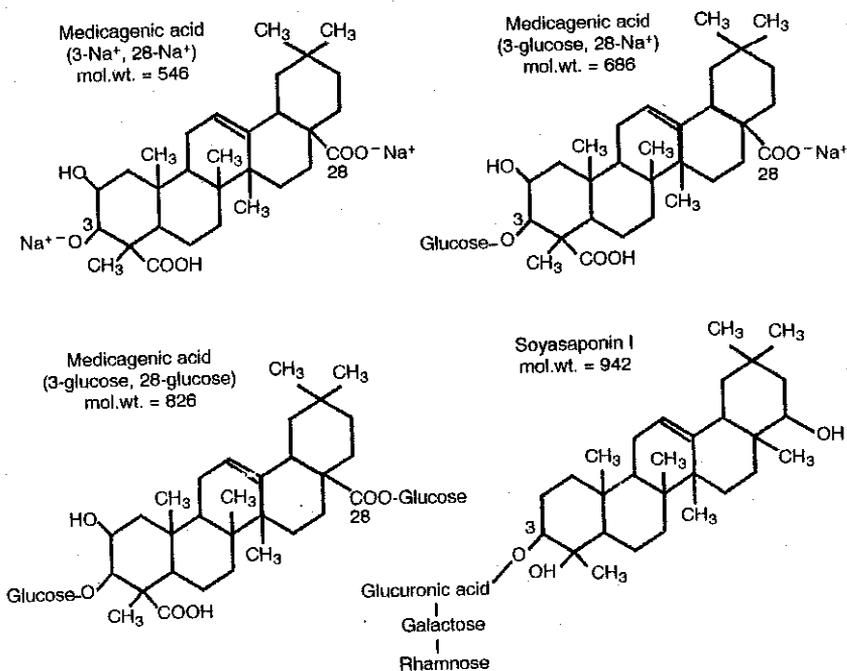
Chemicals

Saponins were isolated from alfalfa roots using methanol extraction and purified using high-resolution preparative chromatography as described elsewhere (Oleszek, 1993; Nowacka and Oleszek, 1994). Certain compounds, MA-3,28Na and MA-3glu,28Na were used as di- and mono-sodium salts, respectively, to enhance solubility for these bioassays. The chemical structures of alfalfa saponin used in the present study are summarized in Figure 1. Sodium salts were prepared by dissolving the purified saponin or sapogenin (100 mg) in methanol (5 mL). Then, sodium hydroxide (10 mL, 5%) was added with continuous stirring. After equilibration, excess water (100 mL) was added to the solution and it was loaded on C-18 column (2 cm × 3 cm), conditioned with distilled water. The column was then washed with water until the eluate pH was neutral. The sapogenin/saponin sodium salt was eluted from the column with methanol, and evaporation of the methanol yielded the sodium salt powder. All other chemicals were obtained from Sigma Chemicals (St. Louis, MO).

Bacterial Strains and Treatment

Bacterial strains used in these studies (Table 1) have been described elsewhere (Zablotowicz, Hoagland, and Wagner, 1996). Cultures were maintained as frozen glycerol stocks. For experimental protocols, all strains were grown on tryptic soy broth on a rotary-shaking incubator (30°C at 100 rpm). After 24 h growth, cells were harvested by centrifugation (8,000 × g, 10

FIGURE 1



min), washed three times in potassium phosphate buffer (KPi, 50 mM, pH 6.8), and resuspended to yield a concentrated suspension of optical density = 8.0 (660 nm). Cell suspensions (3.8 mL) were placed in sterile 25 mL Corex centrifuge tubes (four replicate tubes per treatment) and treated with ethanolic solutions (80% ethanol, 10 mM) of the saponins to attain a concentration of 500 mM in 2% ethanol. Controls received only ethanol. Following treatment, cell suspensions were incubated (30°C at 100 rpm) for 24 h and then assayed for enzyme activity and protein release.

Enzymological and Physiological Assays

Based upon previous research (Zablotowicz, Hoagland, and Wagner, 1996) we investigated three parameters: fluorescein diacetate (FDA) hydrolysis; triphenyltetrazolium chloride (TTC) dehydrogenase activity, and cellular protein leakage after 24 h exposure to the saponins. Cellular leakage or exudation of protein was determined in cell supernatants following centrifugation (14,000 × g, 10 min). Each supernatant was carefully removed and assayed

TABLE 1. Species of rhizosphere organisms tested.

Genus species	Strain	Source
Gram-Negative Bacteria		
<i>Agrobacterium tumefaciens</i>	A-136	Rhizosphere bacterium; Walter Ream, Oregon State University.
<i>Pseudomonas fluorescens</i>	RA-2	Rice rhizosphere (Hoagland and Zablotowicz, 1995)
Gram-Positive Bacteria		
<i>Bacillus thuringiensis</i>	HD-2	Soil bacterium; USDA-ARS NRRL, Peoria, IL
<i>Bacillus thuringiensis</i>	UZ404	Soybean rhizosphere; Zablotowicz et al., 1991
<i>Curtobacterium flaccumfaciens</i>	JM1011	Cotton Rhizosphere; J. McInroy, Auburn University

for FDA activity and protein content. Protein in supernatants was determined using the Bradford reagent (Bradford, 1976).

FDA is a general substrate for several hydrolytic enzymes and serves as substrate for esterases, lipases and certain proteases (Guilbault and Kramer, 1964; Medzon and Brady, 1969). Its activity was determined spectrophotometrically measuring the product of hydrolysis (fluorescein). The assay consisted of 750 μL of cells (or extracellular supernatant), 750 μL KPi buffer (0.1 M, pH 7.6) and 40 μL FDA (2 $\text{mg} \cdot \text{mL}^{-1}$ in acetone). The assay mixture was vortexed and incubated at 30°C for 5 to 60 min depending on activity. The assay was terminated by extraction with acetone (1.5 mL), followed by centrifugation (14,000 \times g, 5 min). The optical density of each supernatant was measured at 490 nm and the total amount of product formed was calculated, based upon an extinction coefficient of 80.3 $\text{mM}^{-1} \cdot \text{cm}^{-1}$.

TTC-dehydrogenase activity can be used as an estimation of respiratory activity or electron transport system potential. Cells (1.0 ml) plus 50 μl TTC (10%) were incubated for 1 h at 30°C. The reactions were terminated/extracted with 9.0 ml MeOH, and the optical density at 485 nm was determined (Casida, Klein, and Santoro, 1964). The effects of various concentrations (50 to 500 μM) of medicagenic acid (3, 28 Na salt) on TTC-dehydrogenase and FDA-hydrolytic activity of *Bacillus thuringiensis* strain HD-2 was assessed using methodology described above.

RESULTS AND DISCUSSION

Protein Leakage

All saponin treatments increased extracellular protein content, except Soyasaponin I (Table 2). The greatest effects were caused by MA-3,28Na and by two of its glucosylated derivatives, MA-3glu,28Na and MA-3,28glu. In general the aglycone (MA-3,28Na) was as active as the mono-glucosylated derivative (MA-3glu,28Na). However, the di-glucosylated derivative (MA-3,28glu), generally had less activity than sodium salt of the aglycone (MA-3,28Na). Aglycones are formed when the sugar has been removed and Na added. Extreme leakage of cellular protein as evidenced in both *Bacillus* strains may be indicative of cell death, although sporulation may be a possible explanation for protein leakage in this bacterial genera. Although statistically significant effects on extracellular protein were observed in response to Soyasaponin I, these effects were minimal in comparison to MA and its derivatives (Table 2). In other studies glycyrrhetic acid, and to a lesser extent β -escin elicited cellular leakage from several rhizobacterial strains described in this study, while betulin and hecogenin had no effect (Zablutowicz, Hoagland, and Wagner, 1996). The magnitude of leakage elicited by MA-3,28Na and MA-3glu,28Na was of a similar magnitude to that elicited by glycyrrhetic acid.

TABLE 2. Effect of alfalfa saponins (500 μ M) on extracellular protein production by rhizosphere bacteria after 24 h exposure.

	Untreated	Soyasaponin I	MA 3,28glu	MA 3glu,28Na	MA 3,28Na
	Protein (ng \cdot mL ⁻¹)				
<i>P. fluorescens</i> RA-2	76 d ¹	62 e	102 c	141 a	111 b
<i>A. tumefaciens</i> A-136	5 e	11 d	17 c	53 a	35 b
<i>B. thuringiensis</i> HD-2	94 d	66 e	166 c	489 b	558 a
<i>B. thuringiensis</i> UZ-404	67 d	28 e	112 c	458 b	507 a
<i>C. flacumafaciens</i> JM-1011	12 d	24 c	27 c	92 a	59 b

¹ Means of 4 replicates, means followed by the same letter do not differ at the 95% confidence level.

TCC-Dehydrogenase Activity

Saponins had little effect on TCC-dehydrogenase activity in the two gram-negative strains (RA-2 and A-136), and in the gram-positive strain *C. flacumafaciens* JM1011 (Table 3). MA-3,28Na and its mono-glucosylated derivative (MA-3glu,28Na) elicited drastic reductions of dehydrogenase activity in both *B. thuringiensis* strains. This reduction represents an overall loss of respiratory activity, and the substantial reduction in the two *Bacillus* strains is further evidence for cell death.

FDA Hydrolytic Activity (Esterase, Lipase, Protease)

Whole-cell FDA activities in *B. thuringiensis* strains were the most affected by saponins; i.e., MA-3glu,28Na and MA-3,28Na reduced activity in whole cells by 80-95% (Table 4). In *C. flacumafaciens*, significant reductions of whole-cell FDA activity was observed in cells treated with soyasaponin I, and both glycosylated MA derivatives. Treatment of RA-2 with soyasaponin I doubled cellular FDA activity compared to untreated cells. In other studies, β -escin increased FDA activity in strain RA-2 by 3.5 to 5-fold compared to untreated cell activity (Zablotowicz, Hoagland, and Wagner, 1996).

In both *B. thuringiensis* strains, extracellular FDA activity was most affected by MA-3,28Na and its glucosylated derivatives (Table 4). In these

TABLE 3. Effect of alfalfa saponins (500 μ M) on triphenyltetrazolium (TTC) activity in rhizosphere bacteria after 24 h exposure.

	Untreated	Soyasaponin I	MA 3,28glu	MA 3glu,28Na	MA 3,28Na
Whole Cells Activity (nmol \cdot mL ⁻¹ \cdot h ⁻¹)					
<i>P. fluorescens</i> RA-2	56 a ¹	55 a	58 a	58 a	60 a
<i>A. tumefaciens</i> A-136	38 b	39 b	41 b	41 b	50 a
<i>B. thuringiensis</i> HD-2	1050 a	1028 a	882 b	191 c	9 d
<i>B. thuringiensis</i> UZ-404	465 b	705 a	557 b	13 c	2 c
<i>C. flacumafaciens</i> JM-1011	92 b	105 ab	117 a	89 c	44 d

¹ Means of 4 replicates, means followed by the same letter do not differ at the 95% confidence level.

TABLE 4. Effect of alfalfa saponins (500 μ M) on cellular and extracellular fluorescein diacetate (FDA) hydrolysis by rhizosphere bacteria after 24 h exposure.

	Untreated	Soyasaponin I	MA 3,28glu	MA 3glu,28Na	MA 3,28Na
Cellular Activity ($\text{nmol} \cdot \text{mL}^{-1} \cdot \text{h}^{-1}$)					
<i>P. fluorescens</i> RA-2	552 b [†]	1034 a	560 b	581 b	586 b
<i>A. tumefaciens</i> A-136	786 a	750 a	817 a	815 a	819 a
<i>B. thuringiensis</i> HD-2	982 a	704 c	1044 a	169 d	48 f
<i>B. thuringiensis</i> UZ-404	4536 a	4269 ab	4070 b	212 c	187
<i>C. flaccumafaciens</i> JM-1011	405 a	303 c	354 b	364 b	390 a
Extracellular Activity ($\text{nmol} \cdot \text{mL}^{-1} \cdot \text{h}^{-1}$)					
<i>P. fluorescens</i> RA-2	36 b	8 c	39 b	49 a	53 a
<i>A. tumefaciens</i> A-136	2 c	3 b	4 a	4 a	3 b
<i>B. thuringiensis</i> HD-2	24 d	14 d	44 c	203 b	437 a
<i>B. thuringiensis</i> UZ-404	349 d	183 e	545 c	1724 b	1884 a
<i>C. flaccumafaciens</i> JM-1011	59 b	29 c	35 c	35 c	167 a

[†] Means of 4 replicates, means followed by the same letter do not differ at the 95% confidence level.

strains the highest levels of extracellular FDA hydrolysis were caused by MA-3,28Na, moderate levels by the mono-glucosylated, and the lowest levels by the di-glucosylated MA. Extracellular FDA activity was also about 2.5-fold greater than in untreated *C. flaccumafaciens* cells incubated with MA-3,28Na, while levels lower than in untreated cells occurred by treatments with di- and mono-glucosylated MA, or soyasaponin I. FDA activity in either whole-cell or extracellular assays of *Agrobacterium tumefaciens* strain A136 was not affected by any of the four compounds tested (Table 4). In our

studies, only a minimal effect of alfalfa saponins was observed on enzymatic activity of resting cells of *A. tumefaciens*. In other studies, the growth of *A. tumefaciens* was severely inhibited by β -escin, but enzymatic activity was not altered (Zablotowicz et al., 1996).

***Effect of MA-3,28Na Concentration
on Bacillus thuringiensis strain HD-2***

The effects of a 24 h exposure of various concentrations of disodium MA extracellular protein and enzyme activities of *B. thuringiensis* strain HD are presented in Table 5. Extracellular protein increased about 2-fold at 50 μ M MA-3,28Na, and was about 4-fold greater at concentrations from 125 to 500 μ M. TTC-dehydrogenase activity was more sensitive to this compound than cellular FDA activity, i.e., a 59% and 25% loss in activity, respectively, occurred at 50 μ M. At 500 μ M, virtually no dehydrogenase activity was present, while cellular FDA hydrolytic activity was about 18% of that in untreated cells of this strain. A linear increase in extracellular FDA hydrolytic activity was exhibited at concentrations of 50 to 500 μ M MA-3,28Na (Table 5).

CONCLUSIONS

These studies demonstrate differential physiological responses of various bacterial genera to structurally different alfalfa saponins. Leakage/exudation

TABLE 5. Extracellular protein, TTC-dehydrogenase activity, and cellular and extracellular fluorescein diacetate (FDA) hydrolytic activity of *Bacillus thuringiensis* HD-2 suspensions as affected by various concentrations of medicagenic acid (3,28 sodium salt), after 24 h exposure.

Concentration (μ M)	Protein (ng \cdot mL ⁻¹)	TTC-dehydrogenase nmol \cdot mL ⁻¹ h ⁻¹	FDA-hydrolysis nmol \cdot mL ⁻¹ h ⁻¹	
	extracellular	cellular	cellular	extracellular
0	127 a ¹	532 a	2226 a	298 a
50	277 b	223 b	1668 b	296 a
125	458 c	35 c	1227 c	377 b
250	498 c	14 c	653 d	469 c
500	547 c	9 c	390 e	613 d

¹ Mean four replicates, means followed by the same letter do not differ at the 95% confidence level.

of protein was observed in all five strains in response to medicagenic acid (MA) and its derivatives. The extraordinarily high cellular leakage of protein in strains UZ404 and HD-2 (both gram-positive, *B. thuringiensis*), correlated directly with loss of their whole-cell enzyme activities. Increased protein excretion may be associated with either autolysis or exudation. However, considering the magnitude of the protein release from these *Bacillus* strains, death, or perhaps sporulation (a distinctive characteristic of *Bacillus*) may have occurred. During the sporulation process, several extracellular enzymes are known to be excreted, e.g., proteases, amylases, RNA- and DNA-degrading phosphodiesterases, and cell wall-lytic enzymes (Schaeffer, 1969). This may explain the increased extracellular FDA-hydrolytic activity found in *B. thuringiensis* following exposure to MA-3,28Na and MA-3glu, 28Na.

A major site of action of saponins is their interference with membrane integrity, especially interactions with sterols (Bangham and Horne, 1962). Thus, bacteria and certain fungi (Phycomycetes, e.g., *Phytophthora*) should be less sensitive to saponins since their membranes are low in sterols. Recent studies comparing medicagenic acid-sensitive and acid-resistant *Trichoderma viride* strains indicated that fatty acid composition is as important as sterol content with regard to saponin sensitivity (Gruiz, 1996). Although the mechanism is unknown, based upon data presented here it is clear that certain saponins-glycosides and their aglycones (such as MA and its derivatives) can affect membrane leakage of gram-negative and gram-positive bacteria.

Various *in vitro* studies have demonstrated effects of saponins from alfalfa on other crops and microorganisms. Some saponins exhibit fungal inhibition at very low concentrations. For example, certain MA glycosides inhibited *Trichoderma viride* growth by 100% at a saponin concentration of 1.6 ppm (Oleszek et al., 1990), and were also highly inhibitory to several species of medically important yeasts at 3 to 15 ppm (Polacheck et al., 1986). Although the *B. thuringiensis* strains were the most sensitive of the bacteria we evaluated, only moderate inhibition of vital activity occurred at 50 μ M MA-3,28Na. Other alfalfa saponins (13 glycosides from roots; 10 from leaves) were examined for growth inhibition of several phytopathogenic bacteria (Timbekova, Isaev, and Abubakirov, 1996). Almost all alfalfa saponins, except a hederagenin analog, suppressed growth of *Corynebacterium michiganense* and *A. tumefaciens*. Growth of the alfalfa pathogen *C. insidiosum*, was suppressed by saponin mixtures from alfalfa roots, purified MA-3glu, and a hederagenin analog. None of the saponins affected the soft rot bacterium *Erwinia carotovora*, and only leaf saponins were inhibitory to *Xanthomonas campestris*.

Allelopathic interactions of saponins in nature are comprised of a multitude of factors. Thus, the effects of saponins on both plants and soil/rhizosphere microorganisms must be considered. For example, treatment of soil

with the saponin β -escin, had no significant effect on the total bacterial population, but populations of gram-negative bacteria and fluorescent pseudomonads increased (Zablotowicz, Hoagland, and Wagner, 1996). This indicates that gram-positive bacteria are more sensitive to β -escin, compared to gram-negative bacteria. In these same studies, fungal propagules decreased after incubation with β -escin. In similar studies, soil applied β -escin dramatically inhibited emergence and growth of several species, including wheat and the weed barnyard grass (*Echinochloa crus-galli* L. Beauv.) (Hoagland, Zablotowicz, and Reddy, 1996). Nodulation of hemp sesbania (*Sesbania exaltata* [Raf.] Rybd.) was unaffected by β -escin, but soybean (*Glycine max* [L.] Merr.) nodulation was reduced in treated soil. Somewhat contrary to this, the growth of *Bradyrhizobium japonicum* and *Rhizobium meliloti* was drastically inhibited by β -escin relative to other bacterial species tested (Zablotowicz, Hoagland, and Wagner, 1996). Incorporation of alfalfa roots or shoots into soil inhibited wheat (*Triticum aestivum* L.) growth, and soil extracts from this alfalfa-amended soil also inhibited *T. viride* growth (Oleszek and Jurzysta, 1987). This inhibition of both species diminished over time, and was associated with microbial detoxification/metabolism of the saponins.

The overall effects of saponins on various species are complex due to the wide variation of their chemical structures, and the broad range of sensitivity to these compounds exhibited by plants and microorganisms. These data indicate that soil and rhizosphere bacteria can be affected by saponins. The ecological impact of saponins produced by crop plants and weeds on microbial populations and on certain microbial processes in soil warrants further study.

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