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VARIABLE SENSITIVITY OF *TRICHODERMA VIRIDE* TO *MEDICAGO SATIVA* SAPONINS

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Key Word Index—*Medicago sativa*; Leguminosae; alfalfa; saponins; medicagenic acid; *Trichoderma viride*; bioassay.

Abstract—Eight saponins were isolated from alfalfa roots (*Medicago sativa*). The sensitivity of *Trichoderma viride* to the saponin varied with the individual saponin isolate. Seven isolates appeared to contain the aglycone, medicagenic acid, and while the other did not, it inhibited the growth of the fungus at higher concentrations than the other isolates. One pair and a triplet of saponins with divergent R_fs evoked near identical biological responses suggesting structural similarity toxic to *T. viride*.

INTRODUCTION

Alfalfa (*Medicago sativa*) saponins are of great interest and importance because they evoke pronounced detrimental effects on animals, fungi and other microorganisms, insects, seeds, blood and tissue cholesterol, and enzymes [1–3]. By and large the effects observed were made with 'saponin extracts' of alfalfa. Studies using purified saponins were seriously hampered by the limited quantities of the purified compounds [4–6]. Such limitations appear to be due to the manner in which the extracts were obtained and the quantity of contaminants that interfere with the effective separation and isolation of the saponins.

An effective and thorough study of the anti-nutritional activity, physiological effects, biosynthetic routes, and role of saponins in plants is highly dependent on the knowledge of the nature, potency and structure of saponins, especially those containing the aglycone medicagenic acid that appear implicated in a great number of biological responses [6–8].

This study reports the extraction of saponins from alfalfa roots by a simple procedure, their isolation, and measurement of the sensitivity of *Trichoderma viride*, NRRL 13034, to the purified saponins.

RESULTS AND DISCUSSION

Flash chromatography, a pressure driven hybrid of medium and short column chromatography [9], was adapted for the extraction of alfalfa saponins. The extraction was quick and removed all of the active components from the roots. Most importantly, the extract was relatively free of contaminants, thus enhancing and simplifying the subsequent isolation of the active components by preparative TLC. The TLC solvent system, unlike those reported in the literature, was also simplified to just methanol to remove major contaminants with minimal separation of the saponins, and 95% ethanol for final purification and isolation of individual components.

Eight purified saponins were isolated (Table 1). Saponins with R_fs 0.24 and 0.39 accounted for 88% of the total isolate. Fungi are known to be sensitive to saponins possessing the aglycone medicagenic acid [10]. With a concentration of saponins of less than 1 mg/ml of medium, only the growth of *Trichoderma viride* was reduced. Advantage was taken of this sensitivity to determine biologically the concentration of saponins in alfalfa [10, 11]. However, the strain of *Trichoderma viride* used for these earlier determinations was unknown and no longer available [private communication, Utah Agricultural Station, Logan, Utah].

Of three strains of *T. viride* (NRRL 1762, 1829 and 13034) and *T. reesai* NRRL 3652 tested, NRRL 13034 was selected as the most suitable assay organism because the growth and sensitivity to saponins was significantly > NRRL 3652 > NRRL 1762. NRRL 1829 was abandoned because of its irregular branching growth pattern rather than the solid disc pattern of the other strains and that of *T. reesai* NRRL 3652.

Figure 1 shows *Tricoderma viride* was most sensitive to a pair of saponins, R_f 0.39 and 0.49, that inhibited the growth in an identical manner and concentration. The fungus was next most sensitive to three saponins (R_f 0.24,

Table 1. The R_f values*, weights† and % of total isolate of saponins from alfalfa roots

Saponin	R_f	Weight (mg)	% Total isolate
1	0.09	2.7	0.5
2	0.19	8.9	1.5
3	0.24	392.0	67.3
4	0.39	122.0	20.9
5	0.49	12.7	2.2
6	0.54	6.2	1.0
7	0.56	35.9	6.2
8	0.71	2.1	0.4

*Silica gel plates developed with 95% EtOH.
†Saponins obtained from 100 g of freeze dried alfalfa root.

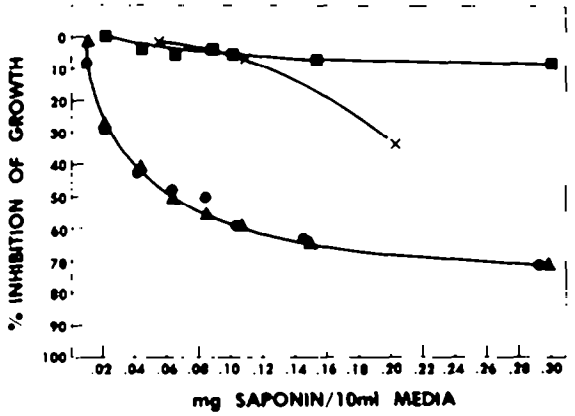

Fig. 1. Growth inhibition of *Trichoderma viride*, NRRL 13034, by alfalfa root saponins: ●, R_f 0.39; ▲, R_f 0.49; ■, R_f 0.19; ×, R_f 0.09. R_fs determined on silica gel plates developed with 95% EtOH.

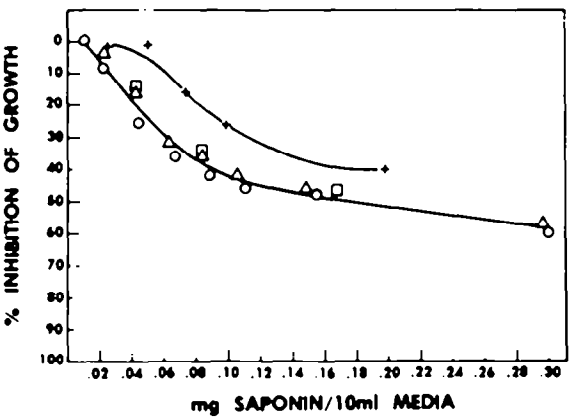

Fig. 2. Growth inhibition of *Trichoderma viride*, NRRL 13034, by alfalfa root saponins: ○, R_f 0.24; △, R_f 0.56; □, R_f 0.71; +, R_f 0.54. R_fs determined on silica gel plates developed with 95% EtOH.

0.56, 0.71) (Fig. 2) that also inhibited growth in an identical manner and concentration. Saponin R_f 0.54 was less active than these followed by R_f 0.09 and R_f 0.19 (Fig. 1).

Clearly, the saponins of divergent R_fs have common structural features for their growth inhibitory activity. Pure medicagenic acid at a concentration of 0.021 mg/plate inhibited growth of *Trichoderma viride* 32%, similar to the response exhibited by saponin R_f 0.09 (Fig. 1). Thus, characterization of these isolated saponins will undoubtedly elucidate those structural features other than its aglycone, medicagenic acid, necessary for its variable growth inhibitory activity.

Foaming, a characteristic attributed to saponins, is considered important in the pathogenesis of ruminant bloat [3]. At concentrations of 0.2 mg/ml H_2O, saponin R_f 0.19 had the most stable foam > R_f 0.09 > R_f 0.24 > R_f 0.56. The others foam, but are not stable for more than a few minutes when examined in the same manner. Of the eight isolated saponins, R_f 0.19 and R_f 0.09 are the least active in inhibiting the growth of *Trichoderma viride*. Thus, individual saponins possess differing activities. Future research into the specific biological activities of saponins can no longer approach the subject on the basis of total saponin content of the varieties or cultivars but must rely on the qualitative differences of individual constituents and their proportional makeup in the plant.

EXPERIMENTAL

Extraction. Flash chromatography [9] was modified and adapted for the extraction of alfalfa root saponins. A column, 33 mm × 53 cm, was packed dry with 50 g of 40 μm silica gel, 1 cm layer of sand, 25 g of freeze dried alfalfa root and topped with 2 cm of sand. Column dead vol. was 140 ml. Under 10–12 psi N_2, the column was eluted sequentially with 7 vols Me_2CO, 3 vols EtOAc, 6 vols EtOH, 3 vols MeOH, 3 vols 70% MeOH, 3 vols 50% MeOH and 3 vols H_2O. Contents from EtOH, MeOH and 70% MeOH eluates inhibited the growth of *Trichoderma viride* NRRL 13034. The active eluates were combined.

Separation and isolation. A two stage prep. TLC removed contaminants and isolated the saponins. In the first stage, the alfalfa root saponin extract streaked on a silica gel prep. TLC plate (20 × 20 cm, 1000 μ thick) was developed with MeOH. Several zones of saponin were detected on a separate TLC plate when sprayed with Stahl's charring reagent and charred at 200°. All coloured zones, corresponding to magenta, purple or blue, were isolated. Each zone was ground with mortar and pestle and extracted with MeOH, filtered through a 0.45 μm filter and concd. Repeated TLC removed more contaminants. In the second stage, the zones were further separated by prep. TLC using 95% EtOH as the developing solvent. Each saponin isolate was further purified by repeated TLC. Analytical TLC was used to check purity and determine the R_f values.

Trichoderma viride *assay.* Five mg of saponin was diluted to 25 ml H_2O. Aliquots from this stock soln were used to make test solns of appropriate concn. Two ml of test soln was added to 8 ml of 3.9% potato dextrose agar (PDA) in a 15 × 150 mm screwcapped tube. Each soln was replicated × 3. The tightly capped tubes were autoclaved 15 min at 15 psi. While hot, the mixture was thoroughly mixed, poured and spread evenly in sterile Petri dishes. When cool, each plate was inoculated with a 6 mm plug of *Trichoderma viride* NRRL 13034 obtained from PDA plates prepared as above and incubated 7 days. The plugs were taken in a radius of 2.5 cm from the inoculum. After the test plates were incubated at 20° for 72 hr, the diameter of growth along three different axes was measured and averaged. Each test was triplicated.

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REFERENCES

1. Cheeke, P. R. (1971) *Can. J. Anim. Sci.* **51**, 621.
2. Birk, Y. and Peri, I. (1980) in *Toxic Constituents of Plant Foodstuff* (Liener, J. E., ed.) 2nd edn, Chap. 6. Academic Press, New York.
3. Bondi, A., Birk, Y. and Gestetner, B. (1973) in *Chemistry and Biochemistry of Herbage* (Butler, G. W. and Bailey, R. W., eds) Vol. 1. Academic Press, New York.
4. Coulson, C. B. and Davies, J. (1962) *J. Sci. Food Agric.* **13**, 53.
5. Pederson, M. W., Zimmer, D. E., Anderson, J. O. and

McGuire, C. F. (1966) in *Proc. X Intern. Grassland Congress*, p. 693.
6. Gestetner, B., Assa, Y., Henis, Y., Birk, Y. and Bondi, A. (1971) *J. Sci. Food Agric.* **22**, 168.
7. Shany, S., Gestetner, B., Birk, Y. and Bondi, A. (1970) *J. Sci. Food Agric.* **21**, 508.
8. Horber, E., Leath, K. T., Berrang, B., Marcarian, V. and Hanson, G. H. (1974) *Entomol. Exp. Appl.* **17**, 410.
9. Still, W. C., Kahn, M. and Mitra, A. (1978) *J. Org. Chem.* **43**, 2923.
10. Zimmer, D. E., Pederson, M. W. and McGuire, C. F. (1967) *Crop Sci.* **7**, 223.
11. Livingston, A. L., Whitehand, L. C. and Kohler, G. O. (1977) *J. Assoc. Off. Anal. Chem.* **60**, 957.