ISOLATION AND BIOLOGICAL ACTIVITY OF THE ROOT SAPONINS OF SIX CULTIVARS OF ALFALFA (*MEDICAGO SATIVA* L.) WITH RESPECT TO DORMANCY AND TIME

By

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LIST OF ABBREVIATIONS AND TERMS

C	Celsius
m	meter
cm	centimeter
mm	millimeter
l	liter
ml	milliliter
µl	microliter
g	gram
mg	milligram
µg	microgram
ng	nanogram
h	hour
min	minute
sec	second
N	Normal
HCl	hydrochloric acid
I.D.	internal diameter
rpm	rotations per minute
ns	not significant
s	significant
T	ton
ha	hectare

lbs	pounds
N	nitrogen
P	phosphorus
K	potassium
NaOH	sodium hydroxide
RT	retention time
CV	cultivar
g/100 ml	grams dry, ground roots per 100 milliliters of water
R_f value	the ratio of the distance that the sample has migrated from the origin to that of the solvent front
origin	the point where the sample is placed on the chromatogram
solvent front	the forward edge of the solvent during the developing of the chromatogram

CHAPTER I

INTRODUCTION

Alfalfa *(Medicago sativa* L.) is an economically important legume crop which yields high protein forage. In Oklahoma, 400,000-500,000 acres of alfalfa are currently being planted and harvested (Sholar *et al.,* 1982; Caddel *et al.,* 1987). It is considered the "queen of the forages" for horse and livestock feeding since protein constitutes up to 20% of its dry weight.

Alfalfa is a potentially long-lived perennial lasting up to 30 years, but most stands are maintained for only four to six years. Four to six cuttings are usually taken from late May through August in Oklahoma. More than 500 cultivars of alfalfa bred for resistance to a host of pests and for time of dormancy are registered in the journal *Crop Science* and approximately 20 new cultivars are introduced each year (Caddel *et al.,* 1987). Alfalfa is highly cross-pollinated, so each plant, even plants in the same cultivar, is genetically different from every other (Sholar *et al.,* 1982).

Alfalfa adds nitrogen and organic matter to the soil, increases the availability of nutrients such as calcium, improves soil structure and increases the rate of water infiltration (Kehr *et al.,* 1983; Caddel *et al.,* 1987). Continuous cropping of alfalfa may deplete soil moisture and lead to the decline of soil nutrients, however.

Reduced yields are commonly associated with old alfalfa stands and alfalfa planted directly after an old stand is plowed down. The yield from a five year old stand previously cropped in alfalfa has been as much as 32% lower than from a stand not previously cropped in alfalfa (Kehr *et al.,* 1983). Even if soil moisture, fertility and pest problems are corrected and alfalfa is reseeded, attempts to increase the production of an

old alfalfa stand are frequently unsuccessful. A reduced yield is also often seen with crops grown in rotation with alfalfa, such as wheat *(Triticum aestivum)*, corn *(Zea mays)*, soybeans *(Glycine max)* and sorghum *(Sorghum bicolor)*.

There is extensive evidence that alfalfa produces allelopathic saponins, which, as residue in soil, may be a major cause of the reduced yields of subsequent crops (Nielsen *et al.,* 1960; Pedersen and Wang, 1971; Leshem and Levin, 1978; Klein and Miller, 1980; Kehr *et al.,* 1983; Hall and Henderlong, 1984; Miller, 1986; Oleszek and Jurzysta, 1987, 1988). Alfalfa saponins have also been shown to possess allelopathic, antinutritional, fungistatic, antimicrobial, choleserolemic and hemolytic properties (Van Atta *et al.,* 1961; Ishaaya *et al.,* 1969; Shany *et al.,* 1970b; Pedersen, 1975; Jurzysta, 1977; Jurzysta, 1979a & b; Malinow *et al.,* 1980; Oleszek and Jurzysta, 1987).

The purpose of this research was to determine, using bioassays: (1), if a relationship existed between the content of and degree of winter dormancy in six cultivars of alfalfa roots, (2), if saponin content varied with time through the seasons, and (3), if saponins were present in a native legume *(Psoralea tenuiflora)* and a naturalized legume *(Lespedeza cuneata)*. The allelopathic activity of alfalfa root saponins of the six cultivars towards a weedy species *(Bromus secalinus*; cheat) as compared to wheat *(Triticum aestivum)* was also measured. The fungistatic activity and saponin content of the test species was measured with the *Trichoderma viride* bioassay. Thin layer chromatography was used to isolate and identify the saponins and the aglycones present in the roots of the six cultivars and the native and naturalized legumes. Capillary gas chromatography was used for the identification and quantification of aglycones present in the plant samples taken in May.

CHAPTER II

LITERATURE REVIEW

Early Studies of Saponins

The earliest scientific studies indicating that phytotoxic substances could be major factors affecting growth rather than physical factors such as competition for light, water, and nutrientsarose in the early 1900s. In 1909, Shreiner and his associates (cited in Miller, 1986) found that some crop plants release growth inhibitors into the soil that make the soils unsuitable for subsequent crops of the same plant. Sigmund (cited in Nord and Van Atta, 1960) produced evidence of an inhibitory effect by certain saponins on germination and seedling development in 1914. Miller reported the presence of saponins in the seed coats and bracts of fourwing saltbush, *Atriplex canescens* in 1919 and Nord and Van Atta confirmed this in 1960. They found that seed germination was depressed in *Atriplex canescens* and antelope bitterbrush *(Purshia tridentata)* treated with 1-5% saponin solutions.

The antifungal activity of alfalfa extracts was first recognized in 1929 by Kofler (cited in Oleszek *et al.,* 1988). Research on plant biochemical interactions expanded rapidly and the term "allelopathy" was coined by Molisch in 1937 to include biochemical interactions, both positive and negative, between plants of all levels of complexity, including microorganisms (cited in Rice, 1984). Mishustin and Naumova (cited in Oleszek *et al.,* 1988) suggested in 1955 that saponins, leached into the soil from alfalfa roots, were allelopathic agents. They reported a decrease in the yield of cotton planted directly after

alfalfa. This decrease was more pronounced after older stands of alfalfa, which led them to suggest that these allelochemicals accumulate in the soil.

Occurrence of Saponins

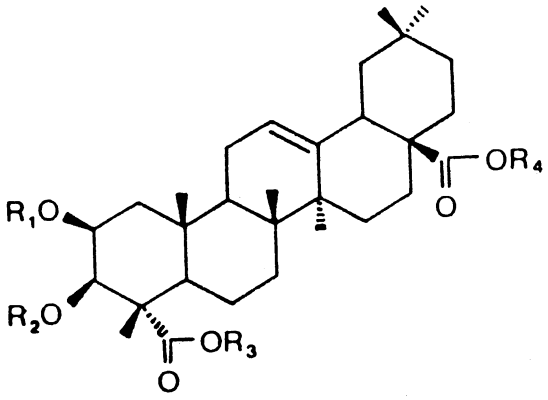
Saponins have been reported in nearly 100 plant families (Price *et al.*, 1987). Most of the families are not commonly used for food or animal feed, but the few that are used are of great importance. Saponin-containing plants used by humans for food, flavoring or health tonics include soya *(Glycine max),* beans *(Phaseolus spp.),* oats *(Avena sativa),* potatoes and eggplant, *(Solanum* spp.), tomatoes *(Lycopersicon escuelentum),* peanuts *(Arachis hypogaea),* red peppers *(Capsicum anuum),* ginseng *(Panex ginseng),* tea *(Thea sinensis),* fenugreek *(Trigonella foenum-graecum),* and liquorice *(Glycyrrhiza glabra)* (Price *et al.*, 1987; Gutsu *et al.*, 1986; Elujoba and Hardman, 1987). Saponin-containing plants used as animal feed or forage include alfalfa, lucerne *(Medicago* spp.), clover *(Trifolium* spp.), and sweet clover (*Melilotus* spp.).

Some Chemical Structures of Alfalfa Saponins

Triterpenes or steroids are C_{30} pentacyclic compounds composed of six isoprene [CH_2=C(CH_3)–CH=CH_2] units (Harborne, 1973). The *in vivo* precursor is isopentenyl pyrophosphate, which is formed from acetate via the mevalonic acid pathway. Triterpenes and triterpenoids are secondary metabolites derived biosynthetically form the acyclic C_{30} hydrocarbon squalene. Saponins are glycosides, composed of carbohydrates and a steroidal or triterpenoidal portion (aglycone).

The aglycones identified from alfalfa saponins are soyasapogenols A and B, hederagenin and medicagenic acid, whose structures are given in Figure 1 (Price *et al.*, 1987). Soyasapogenols C, D, E and F have also been found in hydrolysates of alfalfa, but they have been proven to be artifacts arising from the acid hydrolysis of soyasapogenol B (Jurzysta, 1988; Price *et al.*, 1986).

1



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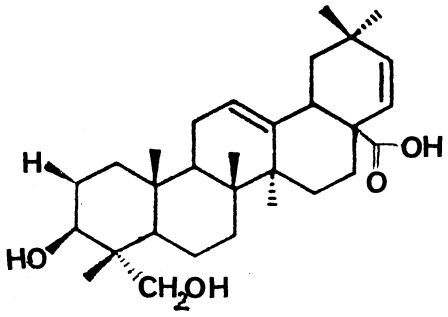

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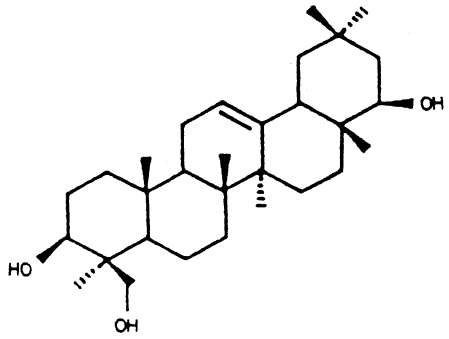


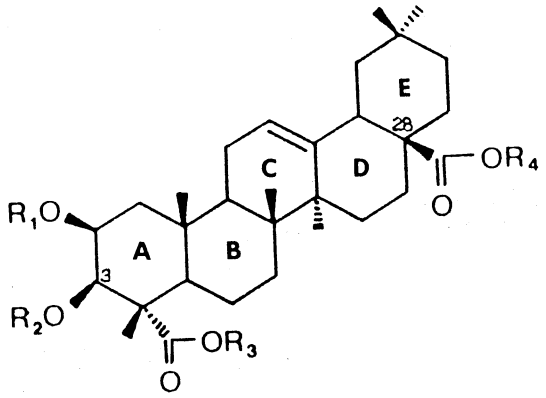
Figure 1. Structures of 1 Medicagenic Acid, 2 Hederagenin and 3 Soyasapogenol B. (Source: Price *et al.*, 1987).

Saponins have been isolated from alfalfa roots, stems, leaves, seeds and flowers (Gestetner, 1971; Oleszek and Jurzysta, 1986). The plant parts differ greatly in the type and quantity of aglycones present. Medicagenic acid glycosides dominate the saponins found in alfalfa roots (Gestetner *et al.*, 1970; Oleszek and Jurzysta, 1986), yet only glycosides of soyasapogenol B and hederagenin found in alfalfa seeds (Oleszek *et al.*, 1988).

The detailed chemical structure of most saponins is unknown. As many as 30 compounds with very similar polarities may be present in alfalfa parts so separation is difficult (Oleszek *et al.*, 1988). The names, source and structural details of medicagenic acid glycosides isolated from *Medicago* spp. is given in Figure 2. The structure of medicagenic acid 3-O-glucopyranoside, isolated from powdered alfalfa roots, was first determined by Morris *et al.* in 1960. Timbekova and Abubakirov confirmed the identification of this compound in 1984 and also identified a bisdesmoside of medicagenic acid. Gestetner isolated another medicagenic acid glycoside from alfalfa tops in 1971 and Timbekova and Abubakirov isolated two hederagenin glycosides from alfalfa tops in 1985. Sugars identified from alfalfa saponins include glucose, galactose, arabinose, xylose, rhamnose and glucuronic acid (Pedersen and Wang, 1971; Timbekova and Abubakirov, 1986). The decomposition of saponins occurs when individual sugar components are cleaved from the glucosidic chain attached to the aglycone (Oleszek *et al.*, 1988). Saponins become increasingly less water-soluble as sugar components are cleaved, and the aglycone portion itself is insoluble in water.

Phytotoxic Effects of Alfalfa Saponins on Plants

The effects of alfalfa on the establishment and growth of numerous crop and weed species using biological, chemical and field methods have been studied by several workers. Nielsen *et al.* (1960) compared the water extracts of five crop species [timothy *(Phleum pratense)*, oat *(Avena sativa)*, corn *(Zea mays)* and potato *(Solanum tuberosum)*]



Name	Source	R_1	R_2	R_3	R_4	References
Medicagenic acid (–)	Aglycone	H	H	H	H	
3-O-Glucopyranoside of –	Roots	H	glu-	H	H	Morris *et al.* (1961); Oleszek and Jurzysta (1960); Oleszek *et al.* (1988)
3-28-di(O-Glucopyranoside) of –	Roots	H	glu-	H	glu-	Timbekova and Abubakirov (1984); Levy *et al.* (1986); Oleszek (1988)
3-O-[β-Rhamnopyranosyl-β-glucuronic-pyranosyl-β-glucopyranosyl] of –	Flowers	H	rha-gluc acid-glu-	H	H	Morris and Hussey (1965)
3-O-Glucopyranoside-(1→6)-gluco-pyranoside-(1→3)-glucopyranoside of –	Tops, Roots	H	glu-glu-glu	H	H	Gestetner *et al.* (1971)

Figure 2. Glycosides of Medicagenic Acid Isolated from *Medicago sativa.*

and found alfalfa to cause the greatest reduction in germination, shoot and root growth in several test species. They reported that alfalfa seedlings, measured 7 days after planting in sand, were most resistant to alfalfa extracts. The studies were based upon their observation of a link between soil structure and the extent of phytotoxicity. They noticed marked differences in the rate of germination and early growth of a number of plant species planted in different soils, even when the fields were maintained under similar conditions.

Van Atta *et al.* developed a reasonably rapid but complicated chemical method for the estimation of saponins in alfalfa in 1961. An ethanol extract of dried, ground alfalfa meal was filtered repeatedly by passing through activated carbon. Methanol and pyridine were used to elute the saponins adsorbed to the activated carbon, and the residue left after evaporation of the solvents was considered to represent the total saponin fraction of the alfalfa meal.

Hanson *et al.* (1963) were among the first researchers to study the saponin content, measured by Van Atta's method, of alfalfa as related to location, cutting, cultivar and other variables. They found that saponins comprised 1.82-3.10% of the dry matter of alfalfa tops. The Lahontan cultivar had the highest saponin content, followed by Ranger, Buffalo and Vernal. The first cutting of alfalfa was consistently lower than subsequent cuttings. The variation between cuttings suggested that saponin content varies with time. The largest percentage (37.6%) of the plot variation for saponin content as determined by an analysis of variance (ANOVA) was due to the interaction between cutting and the location of the plot, and 18% of the variation was due to the cultivar of alfalfa. Based on these findings, they postulated that saponin content was under genetic and environmental control.

Guenzi *et al.* also compared the phytotoxic effect of saponins of different ages and cultivars of alfalfa forage on corn seedling growth in 1964. The cultivars did not differ,

but extracts were generally phytotoxic, with new growth (25 cm high) and first or second cuttings of alfalfa being most inhibitory.

Pedersen *et al.* (1967), using Van Atta's (1961) chemical method for saponin isolation and quantification, reported a difference in the saponin content of the tops of two cultivars, Du Puits and Lahontan, in 1967. The difference increased as the growing season progressed. Du Puits tops had 26% more saponin than Lahontan tops in the first crop, 44% more in the second crop and 59% in the third crop. They used the *Trichoderma viride* bioassay (described below) to determine any allelopathic effects of Ranger, Uinta, Du Puits and Lahontan cultivars and found no correlation between saponin concentration and growth depression of *T. viride,* which led them to suggest a qualitative difference among alfalfa saponins. Pedersen compared the relative quantity and biological activity of saponins from germinated seeds, roots and foliage of two cultivars in 1975. He confirmed his earlier findings of varietal differences in the saponin quality of the foliage, but reported no difference in the root saponins, which were much more toxic to *T. viride* than foliage saponins. Only 15-20 μg of the butanol extract of the roots were required to inhibit *T. viride* growth by 50%, whereas at least 98 μg of foliage extracts were required for the same amount of growth inhibition. Pedersen also reported that root saponins were most concentrated in the outermost layer of the cortex.

Demonstrated cultivar differences in saponin content led many researchers to experiment with selection for tolerance to saponins so that resistance to autotoxicity could be developed. Goplen and Webster (1969) attempted to select for a strain of alfalfa that could produce good yields when planted on "alfalfa-sick" soils previously grown in alfalfa for five years. They selected vigorous specimens of Vernal that were scattered in fields with 'alfalfa-sick' soils from among affected plants which were stunted, chlorotic and abnormally nodulated. They grew the plants in a greenhouse and the seeds, designated 'Selected Vernal', were planted along with seeds of unselected Vernal and seeds of four other cultivars. The yields of all cultivars were uniformly depressed by 'alfalfa-sick'

soils, indicating that selection for tolerance to the phytotoxic factor (thought to be saponins) in 'alfalfa-sick' soils was unsuccessful. Yields improved only slightly with addition of micro nutrients, which were determined to be the only physical factors affecting yield. This indicated the involvement of phytotoxicity.

McElgunn and Heinrichs (1970) also found that the addition of fertilizer did not change the phytotoxic effect of alfalfa-sick soils. They found that different root-zone temperatures did not affect the condition and confirmed Goplen and Webster's finding that different genotypes of alfalfa do not react differently to 'alfalfa-sick' soils.

The establishment, growth and development of other crops planted after alfalfa is also affected by a phytotoxic factor. Leshem and Levin (1978) reported that the germination, growth and yield of cotton was lower in soil previously planted in alfalfa than in either soil previously planted in wheat or bare soil. They found that substances leached form alfalfa roots inhibited the growth of nitrifying organisms, thereby reducing the formation of NO_3 in the soil. They suggested that saponins were responsible for this effect.

Kehr *et al.* (1983) studied alfalfa establishment and production with continuous alfalfa and following soybeans. Yields of alfalfa planted on land not previously seeded in alfalfa were 34% higher than on land reseeded to alfalfa immediately after an old alfalfa stand was plowed down. Yields were not significantly higher when the land previously cropped in alfalfa was fallowed for nine months before reseeding or when soybeans preceeded alfalfa. This indicated that autotoxicity was caused by alfalfa, but that phytotoxic effects of alfalfa residues did not persist in the soil.

Miller (1983) reported that, of those he surveyed, the best preceding crop for alfalfa establishment was corn, followed by various small grains and soybeans, and the worst preceding crop is alfalfa. His results indicating no major genetic differences among cultivars for autotoxicity concurred with the earlier findings of Goplen and Webster (1970) and McElgunn and Heinrichs (1970). In contrast to reports by other researchers, Miller found no evidence supporting saponins as being phytotoxic to alfalfa. He collected the

root zone soils from high and low saponin cultivars, which were stored in a greenhouse four months before alfalfa seeds were sown. He reported that the growth of alfalfa seedlings was similar when grown in the root zone soils of the high saponin or low saponin cultivars, which led him to dismiss saponins as the potential phytotoxic factor. Based on his observation that there are differences in alfalfa seedling growth between the root zone soils of fall dormant and slightly fall dormant cultivars, he suggested that phytotoxic factors might be associated with fall dormancy.

Oleszek and Jurzysta (1986) isolated saponins from alfalfa roots and separated them into cholesterol precipitable and non-precipitable fractions. The non-precipitable fraction contained glycosides of soyasapogenol and hederagenin and the precipitable fraction contained glycosides of medicagenic acid, which constituted 6% of the root dry matter. The medicagenic acid glycosides inhibited wheat seedling root growth by 50% at concentrations as low as 100 ppm and the soyasapogenol B and hederagenin glycosides inhibited seedling growth at much higher concentrations. Only the medicagenic acid glycosides inhibited the growth of *T. viride*. Assuming that medicagenic acid glycosides are 6% of the 6,000-10,000 kg/ha of alfalfa root dry matter in a three year old stand, they calculated that about 500 kg/ha of phytotoxic medicagenic acid glycosides could be incorporated into the top 20 cm of the soil when an alfalfa stand is plowed down. They suggested that the high biological activity of the medicagenic acid glycosides could be responsible for a poor winter wheat crop planted directly after an alfalfa stand.

Oleszek and Jurzysta (1987) later studied the fate of medicagenic acid glycosides in different soil environments, finding that the inhibition of wheat seedling germination and growth was more pronounced on light than on heavy soils, presumably due to the higher sorption and subsequent deactivation of the glycosides by heavy soils. Wheat seedlings grown in water and alcohol extracts of alfalfa roots as well as crude root saponins experienced browning of the meristimatic region in the tip of the roots followed by the complete decay of the root system. Shoot growth was inhibited to a much lesser extent.

Red clover *(Trifolium pratense)* extracts inhibited wheat seedling germination and growth much less than alfalfa extracts.

When 1% (by weight) powdered alfalfa roots were incorporated into four soil types (loose sand, coarse sand, loamy sand and clay loam), the toxicity of water extracts of the soils to *T. viride* decreased with time. The toxicity decreased much more rapidly in heavy soils as compared to sand. Water extracts from a heavy clay soil were not toxic to *T. viride* immediately after the addition of powdered alfalfa roots, which indicated the sorption and subsequent detoxification of medicagenic acid glycosides. They postulated a second process of deactivation whereby soil microorganisms, mainly fungi, detoxified the medicagenic acid glycosides by gradual hydrolysis of water-soluble sugar chain glycosides into more hydrophobic glycosides and finally into medicagenic acid, which is insoluble in water. Soyasapogenol B and hederagenin glycosides incubated in the four soil types and subsequently recovered were not degraded in the soil, probably because they were less soluble in water and/or they were not toxic to soil microorganisms.

Fungistatic Properties of Alfalfa Saponins

Many fungi are sensitive to alfalfa saponins and researchers have used this property to develop bioassays to measure the saponin content of various plants. Zimmer and his associates developed a bioassay for saponin quantification using the beneficial soil fungus, *Trichoderma viride* in 1967. They isolated saponins from alfalfa stems and leaves using the method of Van Atta *et al.* (1961). Testing the growth of selected fungi in 0.5-2 mg saponin/ml medium, they found *T. viride* to be sensitive to concentrations below 1 mg saponin per ml solution. A high negative correlation between the growth rate of *T. viride* and the concentration of saponins indicated that the quantity of fungistatic component was directly correlated with total saponin percentage. They reported that the *T. viride* bioassay indicated a quantitative and a qualitative difference in the saponins of the four cultivars

tested. Modifications (i.e. Jurzysta, 1979a; Nonaka, 1986) of this bioassay procedure are in use today.

Scardavi and Elliot (cited in Oleszek *et al.*, 1988) reported in 1967 that water solubility, as determined by the sugar chain of the saponin, is an essential condition for biological activity. Gestetner *et al.* (1970) found that the growth of the fungus *Sclerotium rolfsii* was arrested by medicagenic acid glycosides but not by soyasapogenol glycosides, which indicated that fungistatic activity was characteristic only of medicagenic acid glycosides. They found that the highest degree of biological activity was associated with alfalfa roots. They reported that the blockage of the two free carboxyl groups or the one hydroxyl group of medicagenic acid greatly diminished its antifungal activity.

Oleszek *et al.* (1988b) later studied the relationship between antifungal activity, aglycone structure and sugar chain composition. They found that solubility affected the hemolytic ability, but not the fungistatic ability of medicagenic acid glycosides. The findings of Nonaka (1986), however, indicated that features other than the structure of medicagenic acid were responsible for the variable inhibitory activity towards the growth of *T. viride* exhibited by medicagenic acid glycosides. He also found that the sensitivity of different strains of *T. viride* to saponins and aglycones varied. Oleszek *et al.* (1988) performed *T. viride* bioassays on isolated and synthetic saponins and found that blockage of important active sites, as suggested by Gestetner *et al.* (1970), caused a sharp drop in biological activity. Antifungal activity of medicagenic acid and its derivatives depended greatly upon free carboxyl and especially hydroxyl groups. The compounds exhibiting highest activity against *T. viride* growth were medicagenic acid, the sodium salt of medicagenic acid and medicagenic acid 3-O-glucopyranoside, all of which have one free hydroxyl and two free carboxylic acid groups.

Other researchers have found that medicagenic acid 3-O-glucopyranoside was inhibitory to other fungi and yeasts. Levy *et al.* (1986) found it to be active against the plant pathogens *Sclerotium rolfsii, Rhizopus mucco, Aspergillus niger, Phytophthora*

cinnamommi and *Fusarium oxysporum* f. sp. *lycopersici*. Polachek *et al*. (1986) found it was toxic to 10 medically important yeasts, including *Toruloppsis* spp., *Candida* spp. and *Geotrichum candidum*.

A possible mode of action of saponins against microorganisms was described by Assa and associates in 1975, who studied the effects of alfalfa saponins on the growth and lysis of the plasmodium *Physarum polycephalum,* which has a cytoplasmic membrane but lacks a cell wall. They found that cholesterol-precipitable saponins from alfalfa roots interacted with membranal sterols, proteins and phospholipids, causing changes in permeability, growth inhibition and lysis of the plasmodia of *P. polycephalum*. The interactions of saponins with proteins also affected membranal enzymic activities such as malate dehydrogenase and NADH oxidase.

CHAPTER III

MATERIALS AND METHODS

Collection, Characteristics and Preparation of the Samples

Plot Information

Alfalfa root samples were collected from a field located at the Oklahoma State University Agronomy Farm, Payne County (Range 2E, Township 19N, Section 18), Stillwater, Oklahoma. Agronomy personnel divided the farm into thirty-six 3.4 x 5 m plots and planted 20 rows 15 cm apart in September, 1984. Six cultivars of alfalfa were planted with six replications each in a randomized complete block design. The 3m borders were planted in an experimental cultivar of alfalfa. The soil type was a Port fine silty loam [Cumulic Haplustolls (Oklahoma Department of Agriculture, 1987)].

The roots of a native legume, *Psoralea tenuiflora* (scurf pea or wild alfalfa), and a naturalized legume, *Lespedeza cuneata* (sericia), were collected from the Oklahoma State University Ecology Preserve, Payne County (Range 1E, Township 17N, Section 19), Stillwater, Oklahoma. The soil type was a Grainola-Masham-Lucien bouldery, loamy soil [Vertic Haplustalfs (Oklahoma Department of Agriculture, 1987)].

Treatment of the Plots

All treatments (irrigation, fertilizer, herbicide and insecticide application, etc.) were identical for the six cultivars of alfalfa. Table I lists fertilizers, herbicides and insecticides used and the dates applied. Rainfall and temperature data for the sampling period are recorded in Table II.

TABLE I

FERTILIZER AND HERBICIDE APPLICATIONS TO THE ALFALFA PLOTS

Date	Fertilizer or Herbicide	Application Rate, lbs/acre
1-18-84	N-P-K 0:0:62	300
4-7-86	Lorsban 4-E	0.75
10-16-86	Kerb 50%	1
3-17-87	Surflan	1.5
	Siwbar	0.5
4-29-87	N-P-K 0:45:0 (east half)	150
	N-P-K 0:0:62 (west half)	250
7-20-87	Post (mixed with 1 qt/acre Crop Oil)	1.5 pints/acre

Source: O. S. U. Agronomy Farm Records.

TABLE II

RAINFALL AND TEMPERATURE DATA FROM JANUARY TO AUGUST 1988

Month	Precipitation, cm water	Average maximum and minimum temperatures, °C	
		maximum	minimum
January	3.56	6.0	-6.5
February	0.89	10.6	-4.7
March	13.89	15.1	0.8
April	10.64	20.7	6.1
May	7.98	27.0	14.0
June	3.30	32.3	17.8
July	6.76	33.8	21.4
August	2.46	35.4	21.2
Total	49.48		

Source: O. S. U. Agronomy Farm, Stillwater, Oklahoma.

Description of the Cultivars

Table III lists the cultivars planted in 1984, the sources and the dormancy ratings. Dormancy ratings are assigned by the USDA based on the ability of the cultivar to survive cold temperatures and its fall growth habits. The cultivars are listed in order of decreasing winter dormancy with Advantage (a dormancy rating of 4 on a scale of 1 to 8) being the most dormant and Cuf 101 (dormancy rating of 8) the least dormant. The following cultivar descriptions are summarized from Caddel *et al.* (1987) and Hay and Forage Grower (1988). Resistance to various pests is classified as follows: low resistance means 6-14% of plants of the cultivar are resistant, moderate resistance means 15-30% of the plants are resistant, resistant means that 31-50% of the plants are resistant, and high resistance means that more than 50% of the plants are resistant.

Advantage, the most dormant cultivar, was released by Nickerson American Plant Breeders in 1981. The described area of adaptation is the North Central United States and it performed well in Oklahoma, yielding about 1.7 tons of dry matter per acre per harvest. It is highly resistant to Phytophthora root and bacterial wilt and moderately resistant to Fusarium wilt and anthracnose.

WL 318 is a semi-dormant cultivar released by Waterman-Loomis Research, Inc. in 1975. The average yield is about 1.7 tons of dry matter per acre per harvest. WL 318 is highly resistant to the pea aphid, moderately resistant to Phytophthora root rot, anthracnose (race 1), Fusarium wilt and downy mildew, and resistant to the spotted alfalfa aphid and bacterial wilt.

Baron is a semi-dormant cultivar released in 1981 by Nickerson American Plant Breeders. The average yield is about 1.6 tons of dry matter per acre per harvest. This cultivar is highly resistant to the spotted alfalfa aphid, pea aphid and blue alfalfa aphid, moderately resistant to bacterial wilt and anthracnose, and resistant to Phytophthora root rot and Fusarium wilt.

TABLE III

SOURCE AND RELATIVE DORMANCY OF CULTIVARS STUDIED

Name	Source	Dormancy Rating
Advantage	Nickerson American Plant Breeders	4
WL 318	Waterman-Loomis Research, Inc.	5
Baron	Nickerson American Plant Breeders	6
WL 515	Waterman-Loomis Research, Inc.	7
Granada	Nickerson American Plant Breeders	8
Cuf 101	California Agricultural Experiment Station	8

WL 515 was released in 1984 by Waterman-Loomis Research, Inc. Yield data are not available for this cultivar. It is resistant to Fusarium wilt, Phytophthora root rot, the spotted alfalfa aphid and stem nematodes, moderately resistant to the pea aphid, and lowly resistant to bacterial wilt.

Granada was released by Nickerson American Plant Breeders in 1984. Yield data are not available. This cultivar is highly resistant to Verticillium wilt, the spotted alfalfa aphid, pea aphid and blue alfalfa aphid and resistant to Phytophthora root rot.

Cuf 101 is a very non-dormant cultivar released in 1977 by the California Agricultural Experiment Station and the United States Department of Agriculture. The average yield is 1.2 tons of dry matter per acre per harvest. This cultivar is highly resistant to the blue alfalfa aphid, pea aphid, spotted alfalfa aphid and Fusarium wilt.

Sampling Method

The top 10 cm of the tap root and the lateral roots of 20 plants of each cultivar, scattered across each of the six plots (three or four plants per plot), were excavated monthly from January to August 1988. Roots 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. The roots were not originally washed with water to avoid saponin loss. The native and the naturalized legumes were collected from May to August 1988 and treated similarly.

Chemical Reagents, Standards and Test Organisms

All solvents used, except where noted otherwise, were Baker Resi-Analyzed grade, obtained from the J. L. Baker Chemical Company, Phillipsburg, New Jersey. Trimethylsilylating reagents, including Tri-Sil® concentrate and silylation grade pyridine, were obtained from the Pierce Chemical Company, Rockford, Illinois. Diazomethane,

[synthesized as described by Ruehle, Browne and Eisenbraun (1979)] was obtained from Dr. E. J. Eisenbraun of the Oklahoma State University Department of Chemistry. The Liebermann-Burchard spray reagent was prepared according to methods used by Liebermann (1874). Nitrogen, helium, argon and breathing air were manufactured and supplied by Sooner Supplies, Inc., Shawnee, Oklahoma.

Purified alfalfa root saponins, medicagenic acid, soyasapogenol B and hederagenin were a gift from Dr. Marian Jurzysta of the Institute of Soil Science and Plant Cultivation, Pulawy, Poland. He isolated and purified these standards from alfalfa (cv. "Cimarron") in 1988 while working in the laboratory of Dr. George Waller, Oklahoma State University Department of Biochemistry. The *Trichoderma viride* fungus culture was also a gift from Dr. Jurzysta. This culture originally came from Dr. D. Zimmer of the U.S.D.A., A.E.S., Utah State University, Logan, Utah.

Wheat *(Triticum aestivum cv. Pioneer 2157)* seeds used were obtained in March 1985 and stored at 4°C. Cheat *(Bromus secalinus)* seeds were collected locally in 1987 by Dr. Thomas Peeper.

Extraction and Hydrolysis Procedures

Grinding and Initial Extraction of the Roots

The dried roots were ground to pass a 0.6 mm screen in a Wiley Mill (Arthur H. Thomas Co., Philadelphia, Pennsylvania). The dry weight of all samples were recorded each month. Sixteen g of the ground roots of each cultivar were transferred to a 250 ml Erlenmeyer flask and 200 ml of triple distilled water was added. The flasks were covered with aluminum foil and extracted for 2 h on a shaker set at 63.6°C, the heat of slow pasteurization, and 200 rpm.

The extracts were filtered through Whatman No. 1 filter paper in a Buchner funnel with a gentle vacuum applied with a water aspirator. One hundred ml of each extract was

reserved for further extraction and the remainder was used for bioassay. A schematic diagram of the extraction and analysis scheme is presented in Figure 3.

Extraction and Purification Procedure

Aliquots (100 ml) of each aqueous extract were placed in a 500 ml separatory funnel and extracted three times with 40 ml of *n*-butanol saturated with water. The extracts were combined for each cultivar and evaporated to dryness under a fume hood. The residues were dissolved in 5 ml methanol and the solutions were filtered with a 5.0 cc glass syringe (Millipore Corporation, Bedford, Massachusetts), using 0.45 μm, 13 mm, nylon 66 membrane filters (Fisher Scientific, Honeoye Falls, New York). The extracts were transferred to scintillation vials, the solvent was evaporated, and the residue was redissolved in 2.0 ml methanol. These extracts were used to determine the saponins present with one dimensional thin layer chromatography. A purified alfalfa root saponin standard obtained from the Cimarron cultivar was used to monitor procedures.

Acid Hydrolysis of the May Samples

The May samples were selected to undergo acid hydrolysis in order to cleave the sugar moieties from the aglycone portion of the saponin molecule. One ml of the methanol fraction was transferred to a 25 ml round-bottomed flask and the residue was evaporated to dryness under nitrogen. Five ml of 2N HCl were added to the residue and the solution was boiled for 5 h in a reflux system. Five ml of distilled water was added and the mixture was shaken. It was extracted in a 100 ml separatory funnel with 5 ml of ethyl acetate. The eluate was centrifuged at 2400 rpm for 15 minutes. The ethyl acetate fraction was transferred to a 7.0 ml scintillation vial, evaporated, and the remaining solution was returned to the separatory funnel. The acid hydrolysate mixture was extracted twice more with 5.0 ml of ethyl acetate. The extracts were combined, the residue was evaporated to dryness, and then dissolved in 0.5 ml methanol. These fractions were used for the

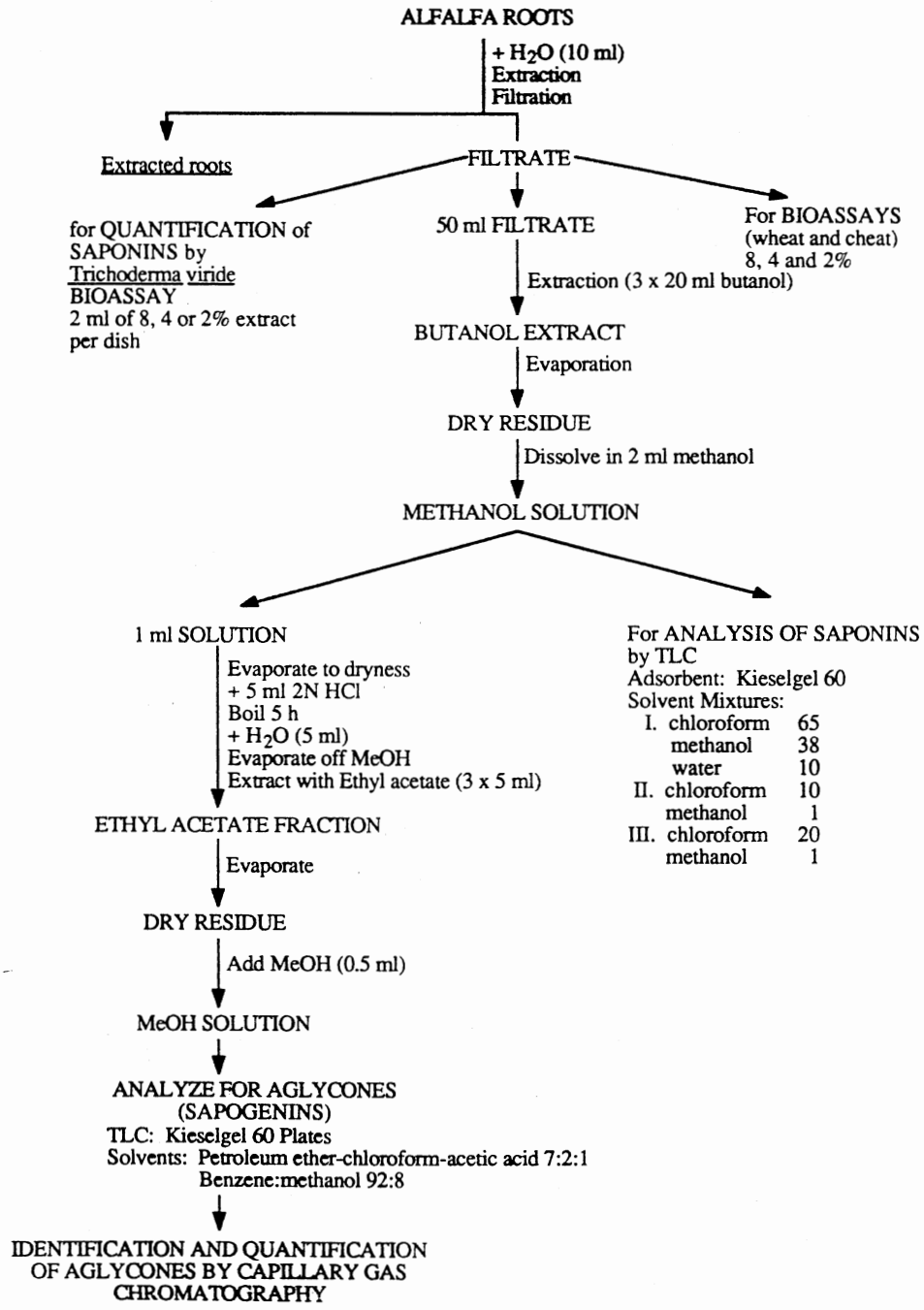


Figure 3. Extraction and Analysis Scheme of Alfalfa Root Saponins.

identification of aglycones using one-dimensional thin layer chromatography, and capillary gas chromatography.

Bioassay Procedures

Choice of Bioassay Method

Carefully selected wheat seeds have a germination frequency of nearly 99%, but cheat seeds have a much lower frequency of germination of less than 60%, as is typical of the seeds of many weedy species. Cheat 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 cheat than in wheat.

Leather and Einhellig (1985) compared the sensitivity of various types of bioassay designs. They found that bioassays involving pregerminated sorghum *(Sorghum bicolor)* seedlings were least sensitive to treatment as compared to bioassays involving ungerminated seeds. It was decided to perform bioassays with pregerminated seedlings so that a large sample size could be maintained and variance could be decreased.

Preliminary comparisons of bioassays involving ungerminated versus pregerminated seeds did not indicate that pregerminated seedlings were less sensitive to a particular treatment, however. No significant difference between the treatments and the control was detected in a bioassay using ungerminated wheat and cheat seeds and 2 ml per dish of alfalfa root water extracts at concentrations of 2, 4 and 8 g/100 ml, whereas differences between the two higher concentrations and the control were highly significant when pregerminated seedlings were tested.

Wheat and Cheat Bioassays

Bioassay experiments were designed to measure the early growth of and cheat treated with plant extracts or purified alfalfa root saponins, as compared to a distilled water

control. The water extracts of the ground alfalfa roots were diluted to one-half and one-fourth strength with distilled water so that concentrations of 8, 4 and 2 g ground roots/100 ml distilled water (hereafter 2, 4 or 8 g/100 ml) were made. Purified alfalfa root saponins were dissolved in distilled water and concentrations of 0.25, 0.5 and 1.0 mg root saponins/ml distilled water were made.

Approximately 75 wheat and cheat seeds were placed on a 9.0 cm disk of Whatman No. 1 qualitative filter paper placed in the lid of an inverted 100 x 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 (20-23°C, 10 h light:14 h dark) for 48 hours. Two additions of 1 ml each were made to each dish to keep the filter paper soaked with distilled water.

After the 48 h incubation, 100 x 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 (having root lengths within 3 mm of each other) were carefully selected and arranged in a radial pattern with the micropylar end toward the center on the premoistened filter paper. Each treatment was replicated four times. The dishes were incubated in the dark at 20°C for 72 hours.

The length of the central root and of the coleoptile of each seedling was measured in mm. Any seedlings showing signs of fungal or bacterial contamination were discarded. Any changes in the appearance of the seedlings, such as root tip browning and necrosis, were also noted. Means per dish and per treatment were calculated and Student's t-tests were used in the analysis (Steel and Torrie, 1985).

Trichoderma viride Bioassays

Bioassay experiments which measure the growth in diameter of colonies of *Trichoderma viride* in the presence of varying concentrations of the alfalfa root water extracts and isolated alfalfa root saponins standards were designed. This fungus is

regarded as a beneficial decomposer in the soil. Colony growth has been shown to be suppressed by saponins by Jurzysta (1979), who modified the original method of Zimmer *et al.* (1967).

Containers used were 100 x 15 mm plastic petri dishes. The potato dextrose agar was made by boiling 200 g of peeled and thinly sliced potatoes in 1 l of distilled water, filtering the potatoes out with cheesecloth, and adding 10 g each of dextrose and agar to the filtrate. Defined amounts of the alfalfa root water extracts or isolated root saponins that corresponded to the amount used per dish in the wheat and cheat bioassay experiments were added to the mixture. An equal amount of distilled water was added to the controls. The contents were mixed and autoclaved 30 min. Each petri dish was filled with 33 ml of the potato-dextrose-agar mixture with or without any other additives. Each treatment was replicated six times.

After the agar cooled and solidified, the dishes were inoculated in the center with 10 mm discs taken from the periphery of a *T. viride* culture which had been grown under controlled conditions (30°C in a dark incubator) for 72 h. The dishes were inverted and incubated 30°C for 48 h. The diameter of the colony was measured in mm. Any dishes exhibiting bacterial or fungal contamination were discarded. The means per treatment was calculated and the data were analyzed with Student's t-tests (Steel and Torrie, 1985).

Thin Layer and Capillary Gas Chromatography

Thin Layer Chromatography

One-dimensional thin layer chromatography was used to identify the saponins from the butanol extracts of all samples and the aglycone portions from the May samples, which had undergone acid hydrolysis. Kieselgel 60 F_{254} plates (10 x 20 cm) with a thickness of 0.25 mm were used. The prepared samples and standards of purified alfalfa root saponins, medicagenic acid, soyasapogenol B and hederagenin were all dissolved in

methanol. A 10 µl syringe (Hamilton Co., Reno, Nevada) was used for spotting the samples and the standards. Two µl was spotted on plates for identification of the saponins and 1 µl for identification of the aglycones. Standards were prepared at a concentration of 1 µg/µl and various amounts were spotted. The solvent systems (all v:v:v) used separately for identification of the saponins were chloroform:methanol:water 65:38:10 (Jurzysta, 1987), chloroform:methanol 20:1 and chloroform:methanol 10:1 (Timbekova and Abubakirov, 1986). The solvent systems used for the identification of the aglycone portion of the May samples were petroleum ether:chloroform:acetic acid 7:2:1 and benzene:methanol 92:8 (Oleszek and Jurzysta, 1987). After developing the solvents were allowed to evaporate from the plates and they were heated on a steam hotplate (~ 80-90°C) for 2 min. The plates were sprayed with Liebermann-Burchard reagent and heated an additional 5 min. Fluorescent spots were visualized under long-wave UV (366 nm). The color of the spots was recorded and R_f values were calculated. Photographs of the plates as seen under UV light were taken with Kodak Ektachrome 400 film.

Capillary Gas Chromatography

The hydrolyzed samples and the standard (cv. "Cimarron") were derivatized prior to analysis. The methods used were similar to those of Jurzysta and Jurzysta (1978), Brawn *et al.*, 1981 and Price *et al.* (1986); however, these scientists used packed columns and the procedure described here is for a capillary gas chromatography column. A microsyringe was used to transfer 20 µl of each sample and standard to an acid-washed 4 ml scintillation vial. An excess of diazomethane (0.5-1.0 ml) was added and the reaction was complete in 20 min., as indicated by the disappearance of the yellow color. The diazomethane and solvent were removed under nitrogen.

A trimethylsilylating reagent was prepared by adding one part of Tri-Sil® concentrate to three parts silylation grade pyridine. Thirty µl of the reagent was added to each sample and the mixture was heated in a heating block at 70°C for 2 h. The samples were then

cooled and evaporated to dryness under argon. Immediately afterwards, 100 µl of HPLC grade isooctane (dry) [2,2,4 trimethylpentane (Aldrich Chemical Company, Milwaukee, Wisconsin)] was added to each sample and standard. The samples and standards were later diluted with an additional 900 µl of dry isooctane. Samples were stored at 4°C and were discarded one week after derivitization.

Analyses were performed on a Varian Model 3300 instrument equipped with a flame ionization detector and a Varian Model 4290 integrator (Varian Instrument Group, Walnut Creek, California). The column was a 30 m x 0.25 mm I.D. DB-fused silica capillary column (J & W Scientific, Folsom, California) with a flow rate of He being 50 cm/sec. Hydrogen and breathing air flow rates were adjusted to give maximum detector response. A capillary syringe was used to inject 1 µl of the solvent (dry isooctane) and 1 µl of the sample or standard. The samples were injected onto a 105°C column, held for 2 min, and the temperature was increased from 105-300°C at 30°C/min. The temperature was held at 300°C for 2 min., and then raised to 320°C at 2°C/min, and held at 320°C for 20 min. The retention time and area of each peak of interest were determined. Peaks with low retention times (less than 20 min.) were not considered as they were considered to be byproducts of the derivatization reactions.

CHAPTER IV

EXPERIMENTAL RESULTS AND DISCUSSION

Sampling Method

The dry weights of the roots collected ranged from 61.2-112.9 g, with a mean of 74.8 g and a variance of 11.0 g. Differences in the weights were due to different proportions of small to large roots and differences in the amount of lateral roots collected. The mean weight per individual cultivar for the sampling period was close to the overall mean.

Rainfall and Temperature Data

The data suggest no consistent relationship between growth inhibition of wheat and cheat and rainfall, though no statistical analysis was done. An inconsistent trend of greater growth of wheat and cheat with lower amounts of rainfall can be observed. The growth in January, June and August, months with low rainfall, is considerably more than other months. This is probably due to factors other than rainfall since growth was not increased in the month with the least rainfall, February. No relationship between temperature and growth inhibition was apparent.

The data suggest a trend of decreased growth of *T. viride* with higher amounts of rainfall. The average growth of *T. viride* is response to extracts of the six cultivars was lowest in March, April and May, months with high rainfall. The average growth of *T. viride* treated with extracts of roots collected in January was greater than or equal to the growth of the control, and the average growth of *T. viride* treated with extracts of roots

collected during other months with low rainfall was at least 85% of the control. No apparent relationship between *T. viride* growth and temperature can be discerned.

Wheat and Cheat Bioassay Results

Statistical Analysis

Table IV contains the results of the Student's t-tests performed each month between the wheat root length means as affected by the water extracts of each cultivar compared to the water extracts of every other cultivar and Table V contains the results of the Student's t-tests performed similarly between the cheat root length means. All differences between the control and the water extracts of all cultivars at both concentrations were highly significant and were not recorded in the tables. Statistical analyses were not performed on the shoot means because of variability and little apparent inhibition. No consistent significant differences between any of the cultivars could be observed. The most marked differences between the cultivars were in March and April, the months with the highest rainfall.

Significant differences between cultivars with respect to wheat root growth were detected in three of eight months. The difference with respect to cheat root growth between Advantage and all other cultivars was highly significant ($P < .001$) during March at a concentration of 2 g/100 ml, but not at a higher concentration of 4 g/100 ml. Similar results were seen in July, with highly significant differences between Advantage and all other varieties except Granada at the low concentration but no significant difference between varieties at the high concentration. August was the only month i which there were significant differences between Advantage and most other varieties at both concentrations. WL 318 and WL 515 were significantly different at a concentration of 2 g/100 ml in March, April, May, June and August. Significant differences between

TABLE IV

RESULTS OF STUDENT'S T-TESTS: MONTHLY COMPARISONS BETWEEN THE WHEAT ROOT LENGTH MEANS AS AFFECTED BY WATER EXTRACTS OF SIX CULTIVARS OF ALFALFA

Variety	Conc., g/100 ml	January B	C	G	3	5	February B	C	G	3	5	Conc., g/100 ml	March B	C	G	3	5	April B	C	G	3	5
A	8	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	4	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
	4	*	ns	*	ns	ns	ns	*	ns	ns	*	2	ns	***	***	**	ns	ns	ns	*	ns	***
B	8		ns	ns	*	ns		ns	ns	ns	ns	4		ns	*	ns	ns		ns	ns	ns	**
	4		*	ns	*	*		ns	ns	ns	ns	2		***	***	***	**		ns	ns	ns	***
C	8			ns	ns	ns			ns	ns	ns	4			ns	ns	*			ns	ns	**
	4			*	ns	ns			ns	ns	ns	2			ns	ns	*			*	ns	***
G	8				ns	ns				ns	ns	4				*	*				ns	*
	4				*	*				ns	*	2				ns	ns				ns	***
3	8					ns					ns	4					ns					*
	4					ns					*	2					ns					***

Variety	Conc., g/100 ml	May B	C	G	3	5	June B	C	G	3	5	July B	C	G	3	5	August B	C	G	3	5
A	4	*	ns	*	*	ns	ns	ns	*	ns	ns	*	*	ns	ns	ns	*	**	*	ns	**
	2	ns	ns	ns	ns	ns	*	ns	ns	ns	ns	**	***	ns	**	***	*	ns	ns	ns	***
B	4		***	ns	ns	ns		ns	ns	ns	ns		ns	*	*	ns		ns	ns	ns	ns
	2		ns	**	ns	ns		*	*	*	ns		ns	**	ns	ns		ns	ns	**	ns
C	4			***	***	***			ns	ns	ns			**	**	**			ns	*	ns
	2			**	ns	ns			ns	ns	ns			ns	ns	ns			ns	ns	*
G	4				ns	ns				ns	ns				*	ns				*	ns
	2				ns	ns				ns	ns				ns	ns				ns	*
3	4					ns					ns					ns					*
	2					ns					ns					ns					***

A = Advantage; B = Baron; C = Cuf 101; G = Granada; 3 = WL 318; 5 = WL 515

* = P <. 05; ** = P < .01; *** = P < .001; ns = not significant

TABLE V

RESULTS OF STUDENT'S T-TESTS: MONTHLY COMPARISONS BETWEEN THE CHEAT ROOT LENGTH MEANS AS AFFECTED BY WATER EXTRACTS OF SIX CULTIVARS OF ALFALFA

Variety	Conc., g/100 ml	January B	January C	January G	January 3	January 5	February B	February C	February G	February 3	February 5	Conc., g/100 ml	March B	March C	March G	March 3	March 5	April B	April C	April G	April 3	April 5
A	8	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	4	ns	ns	**	ns	ns	ns	ns	ns	**	***
	4	ns	ns	*	ns	ns	ns	ns	ns	ns	ns	2	***	***	***	***	***	*	ns	ns	ns	***
B	8		ns	ns	ns	ns		ns	ns	ns	*	4		ns	**	ns	**		ns	ns	*	**
	4		*	ns	ns	ns		*	ns	ns	ns	2		ns	ns	ns	*		**	ns	ns	***
C	8			ns	ns	ns			ns	ns	ns	4			*	**	***			ns	*	***
	4			ns	ns	ns			ns	ns	ns	2			ns	ns	*			**	**	***
G	8				ns	ns				ns	ns	4				***	***				*	**
	4				ns	*				ns	ns	2				ns	*				ns	***
3	8					ns					ns	4					ns					ns
	4					ns					ns	2					ns					***

Variety	Conc., g/100 ml	May B	May C	May G	May 3	May 5	June B	June C	June G	June 3	June 5	July B	July C	July G	July 3	July 5	August B	August C	August G	August 3	August 5
A	4	ns	ns	ns	*	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	**	*	*	**	***
	2	ns	ns	ns	ns	ns	ns	ns	*	ns	*	***	***	ns	***	*	**	***	ns	ns	***
B	4		ns	ns	ns	ns		ns	ns	ns	ns		ns	ns	ns	ns		ns	ns	ns	ns
	2		ns	**	ns	ns		ns	***	ns	ns		ns	***	ns	ns		ns	**	**	ns
C	4			ns	ns	ns			ns	*	ns			ns	ns	ns			ns	ns	*
	2			**	ns	ns			*	*	**			***	ns	ns			***	***	ns
G	4				*	ns				ns	ns				ns	ns				ns	ns
	2				*	*				***	***				ns	ns				ns	***
3	4					ns					ns					ns					ns
	2					ns					ns					ns					**

A = Advantage; B = Baron; C = Cuf 101; G = Granada; 3 = WL 318; 5 = WL 515

* = P <.05; ** = P < .01; *** = P < .001; ns = not significant

cultivars with respect to wheat root growth were not detected in more than three of the eight months.

These findings were consistent with those of Pedersen, who reported no significant difference between root extracts of four cultivars with respect to toxicity to *T. viride*. Guenzi *et al.* (1964) reported no significant difference in the phytotoxicity of the forage extracts of different cultivars of alfalfa to corn seedling roots. Pedersen and others (Hanson *et al.*, 1963), however, have reported differences between cultivars in the saponin content of the forage portion of alfalfa.

Comparison of Wheat and Cheat Growth

Figure 4 depicts wheat and cheat root growth averaged over the aqueous extracts of the cultivars each month. The standard deviation from each of the means was fairly small, indicating similar responses of the wheat and cheat seedling growth to the cultivars. Figures 5-20 depict wheat and cheat root and shoot growth as affected by aqueous extracts from the roots of each cultivar, *L. cuneata* and *P. tenuiflora*. The growth of cheat roots, expressed as a percentage of the control, was consistently less than the growth of the wheat roots. Cheat root growth was inhibited more than wheat root growth by 8-10% for all months except June, which had only a 2% difference in inhibition. Waller and Jurzysta (1988) found that the seeds of other weedy species such as barnyardgrass *Echinochloa crus-galli)*, pigweed *(Amaranthus* sp.) and dandelion *(Taraxacum officinale)* were more inhibited by alfalfa root water extracts than wheat and alfalfa seeds. These data would imply that certain alfalfa saponins are potential herbicides.

The average growth of wheat and cheat roots and shoots treated with extracts of *L. cuneata* and *P. tenuiflora* roots was much greater than the growth of the roots treated with extracts of the six cultivars. The extracts of the native and the naturalized legumes produced similar responses. Root extracts at a concentration of 4 g/100 ml inhibited

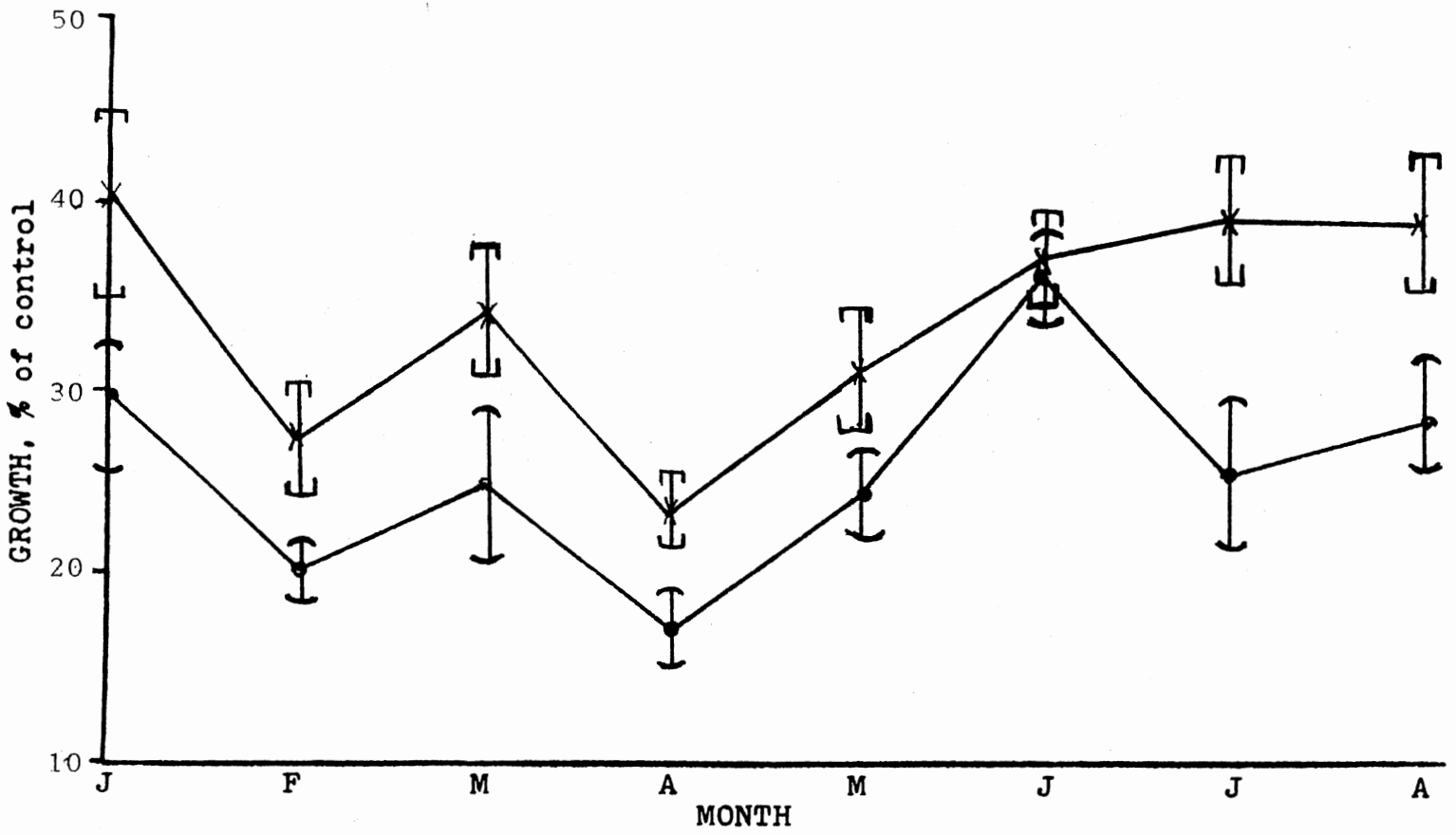


Figure 4. Wheat (×) and Cheat (•) Root Growth Averaged Over Extracts of All Cultivars Each Month with Bars Indicating Standard Deviation.

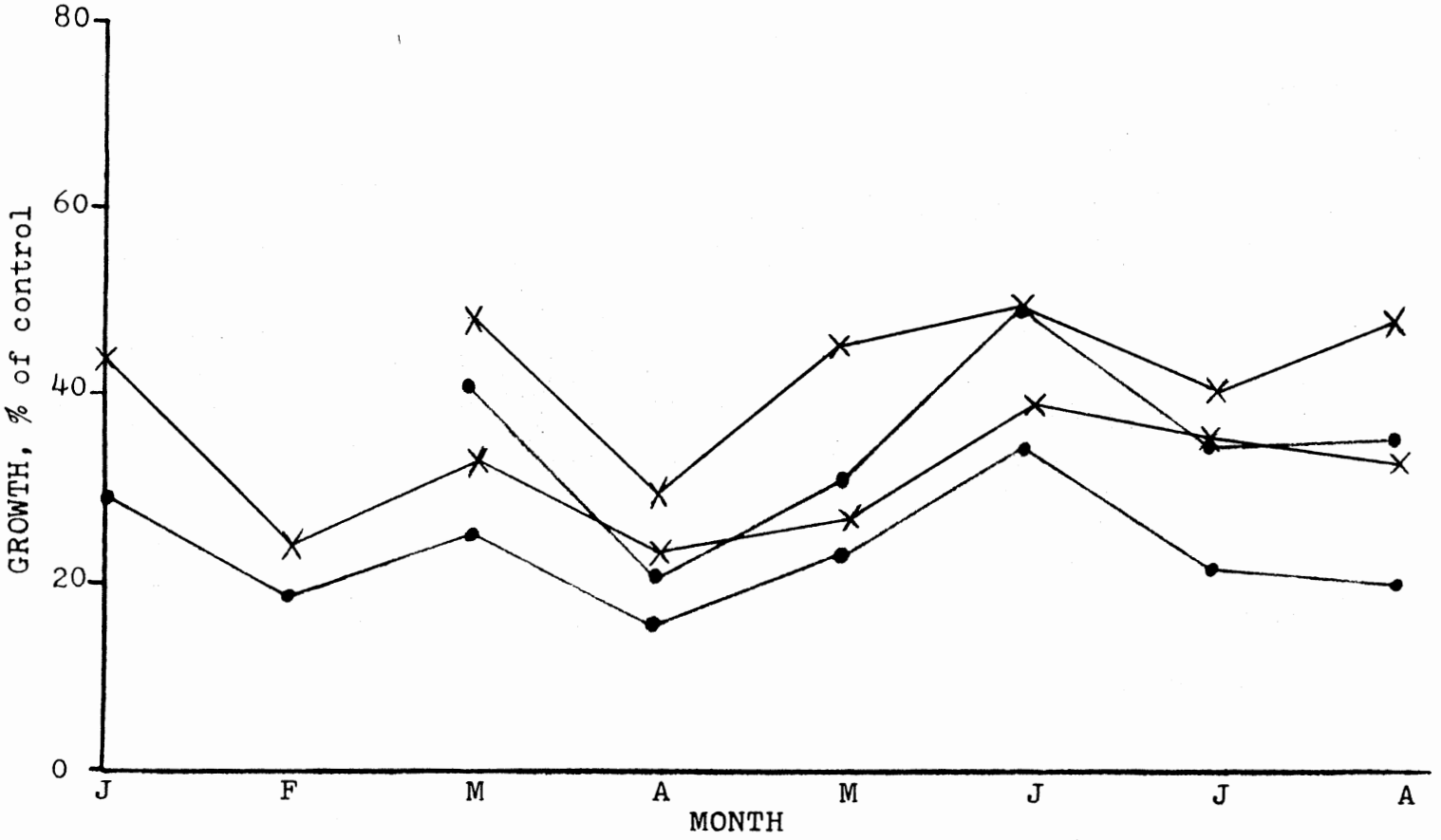


Figure 5. Growth as a Percentage of Control of Wheat (×) and Cheat (•) Roots Treated with Water Extracts of Ground Advantage Roots at Concentrations of 2 g/100 ml (March-August 1988) and 4 g/100 ml (January-August 1988).

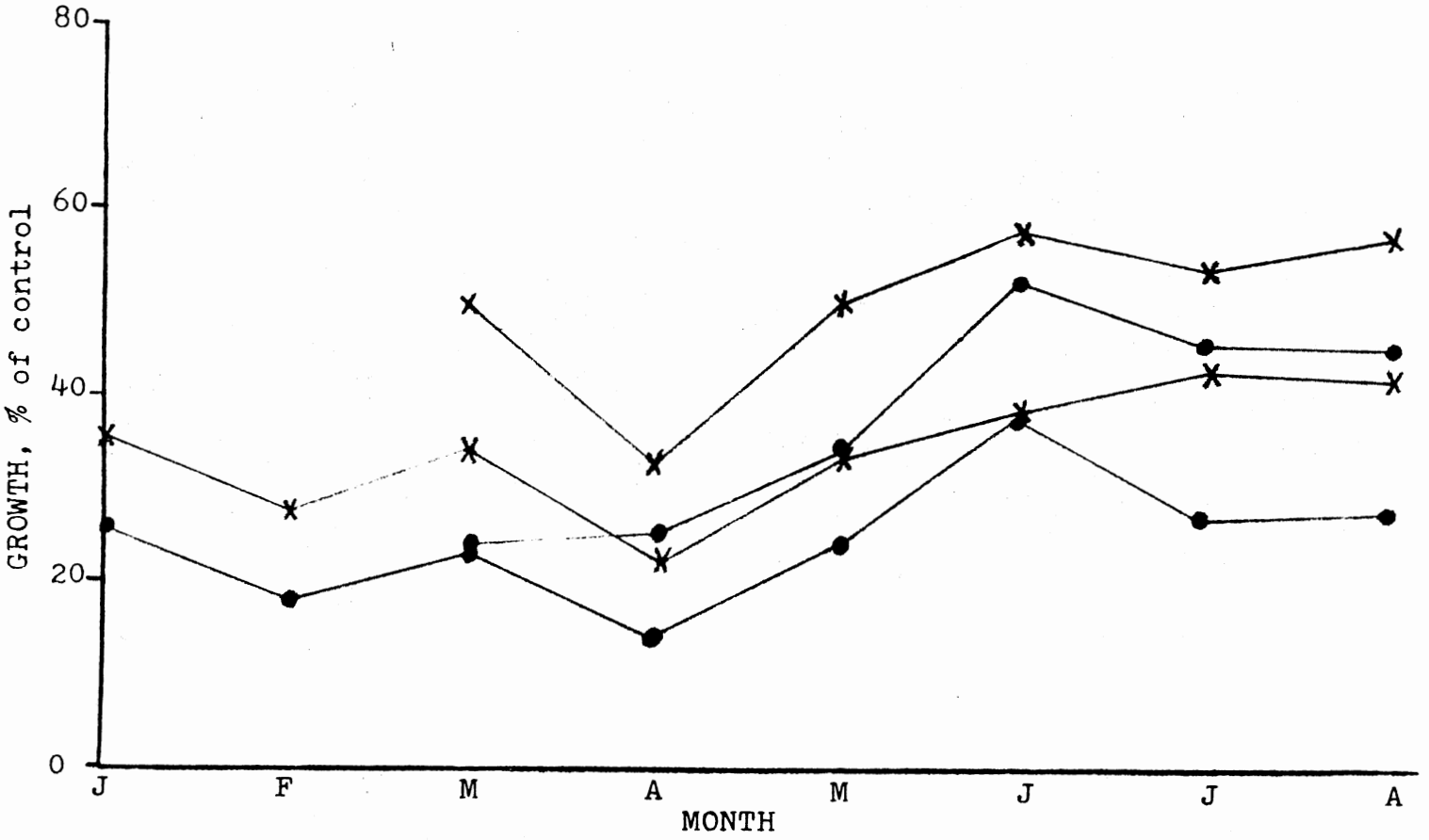


Figure 6. Growth as a Percentage of Control of Wheat (×) and Cheat (•) Roots Treated with Water Extracts of Ground Baron Roots at Concentrations of 2 g/100 ml (March-August 1988) and 4 g/100 ml (January-August 1988).

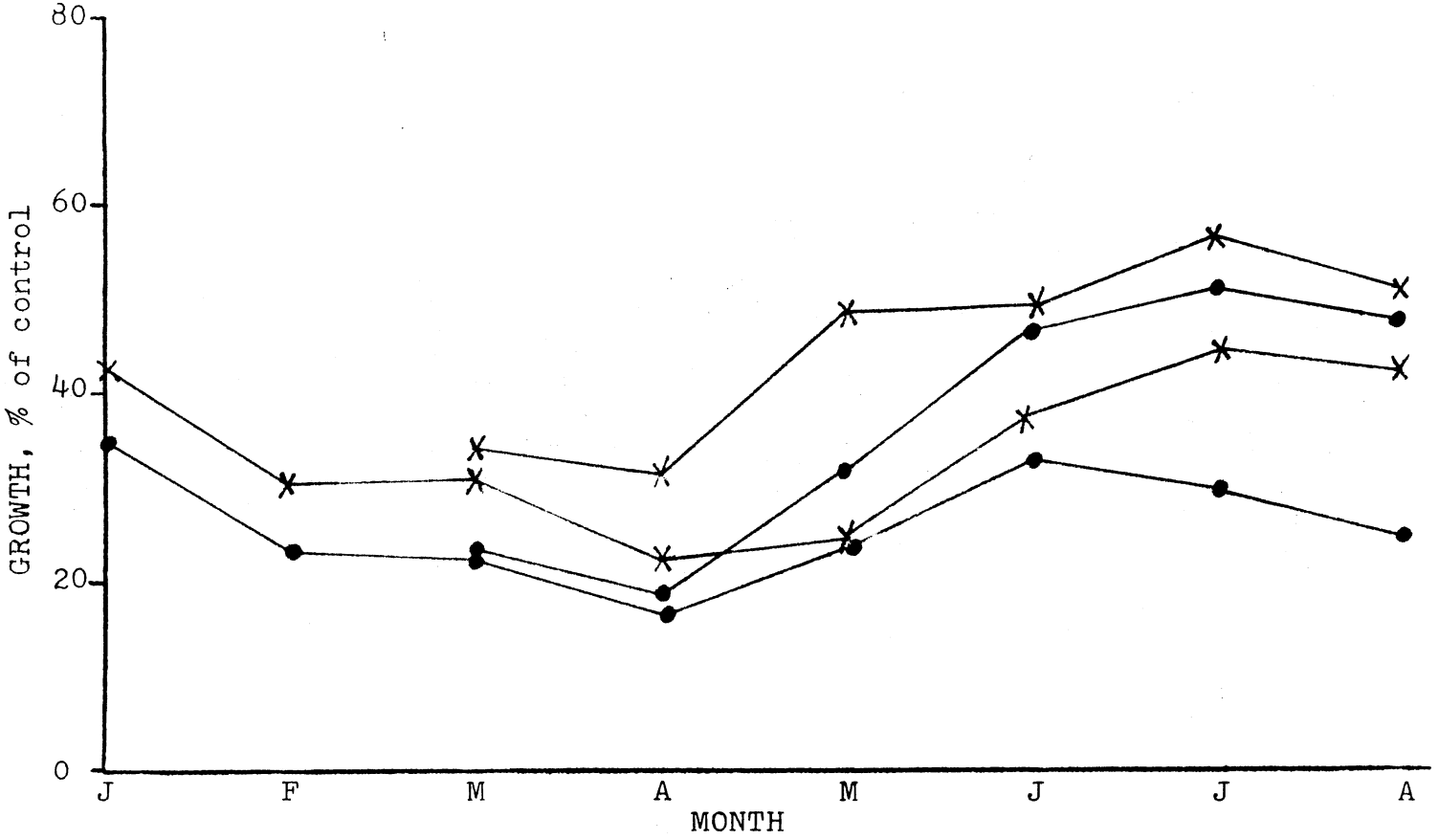


Figure 7. Growth as a Percentage of Control of Wheat (×) and Cheat (•) Roots Treated with Water Extracts of Ground Cf 101 Roots at Concentrations of 2 g/100 ml (March-August 1988) and 4 g/100 ml (January-August 1988).

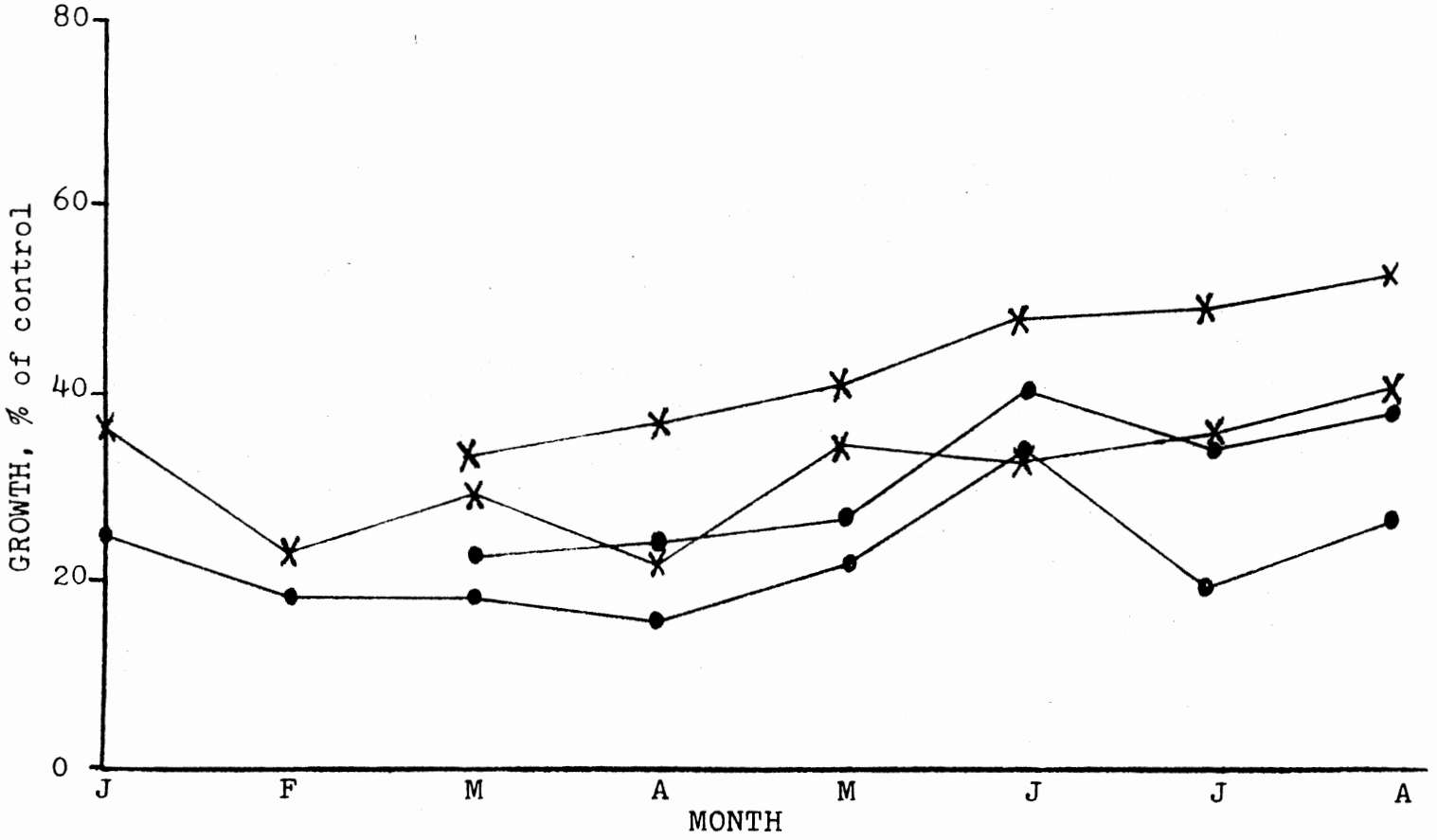


Figure 8. Growth as a Percentage of Control of Wheat (×) and Cheat (•) Roots Treated with Water Extracts of Ground Granada Roots at Concentrations of 2 g/100 ml (March-August 1988) and 4 g/100 ml (January-August 1988).

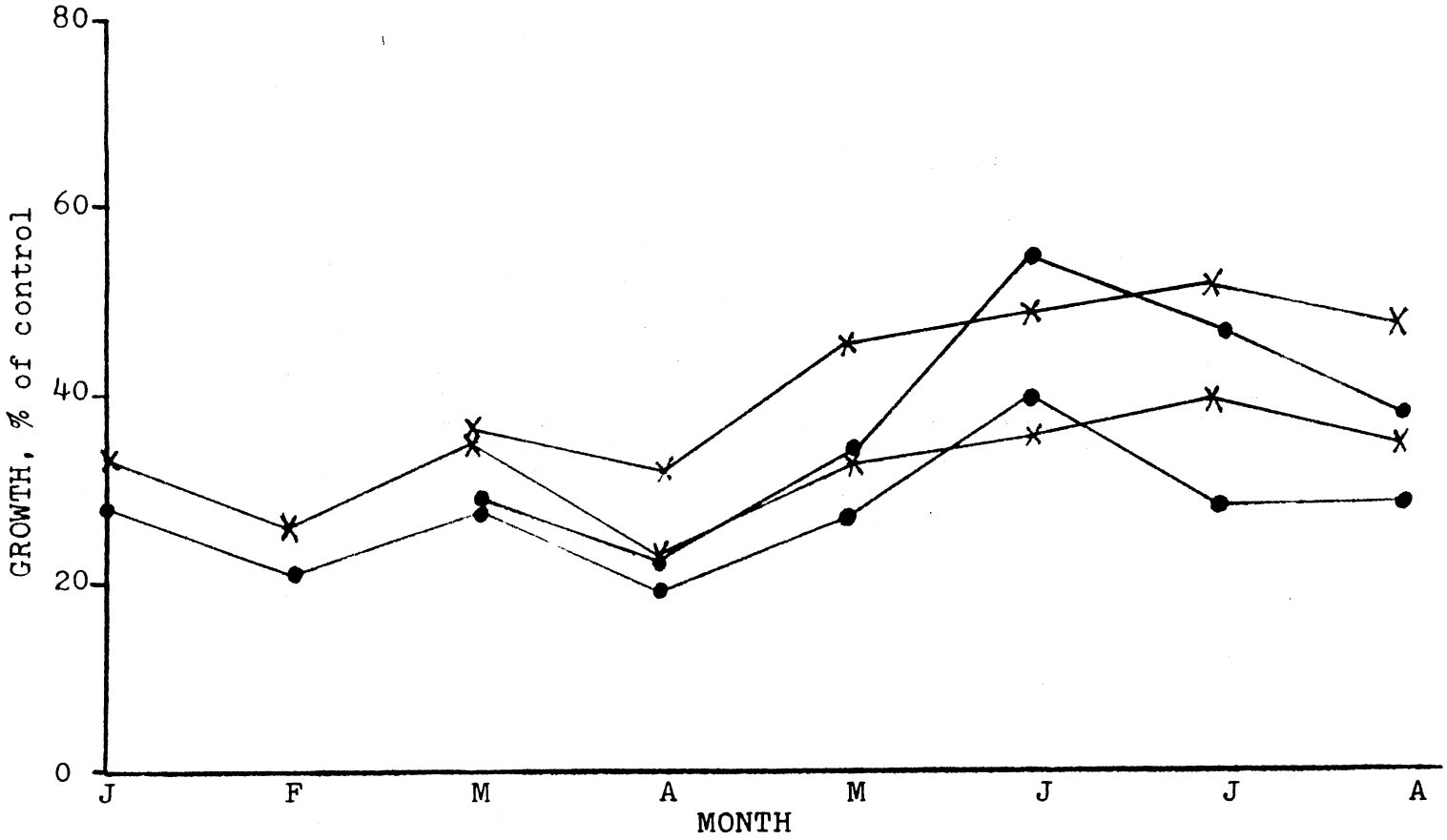


Figure 9. Growth as a Percentage of Control of Wheat (×) and Cheat (•) Roots Treated with Water Extracts of Ground WL 318 Roots at Concentrations of 2 g/100 ml (March-August 1988) and 4 g/100 ml (January-August 1988).

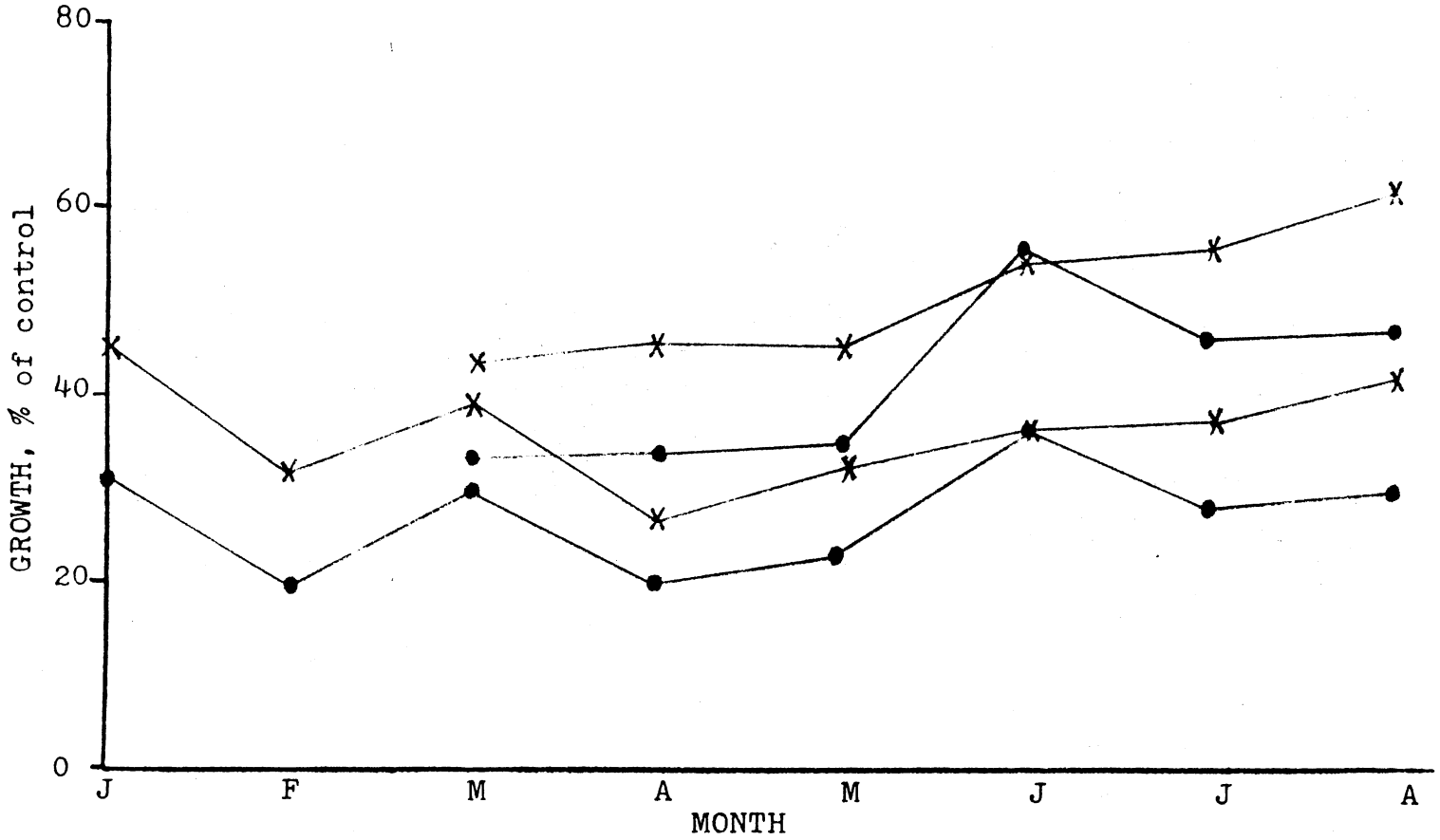


Figure 10. Growth as a Percentage of Control of Wheat (×) and Cheat (•) Roots Treated with Water Extracts of Ground WL 515 Roots at Concentrations of 2 g/100 ml (March-August 1988) and 4 g/100 ml (January-August 1988).

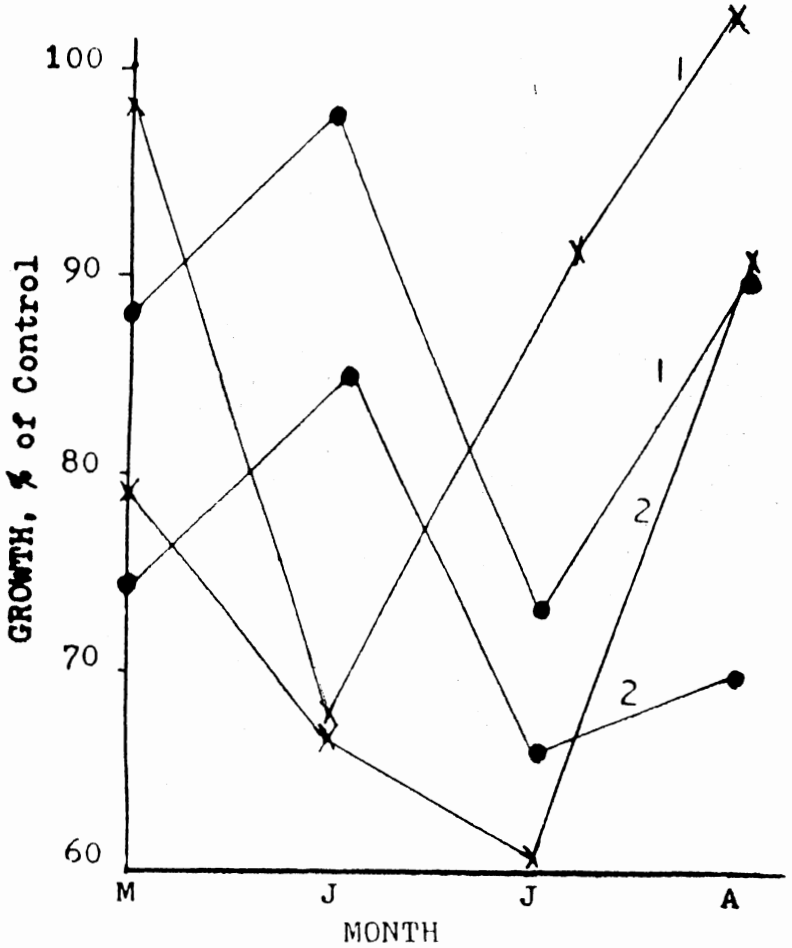


Figure 11. Growth as a Percentage of Control of Wheat (×) and Cheat (•) Shoots Treated with Water Extracts of Ground *Lespedeza cuneata* Roots at Concentrations of 2 g/100 ml (1) and 4 g/100 ml (2) from May to August 1988.

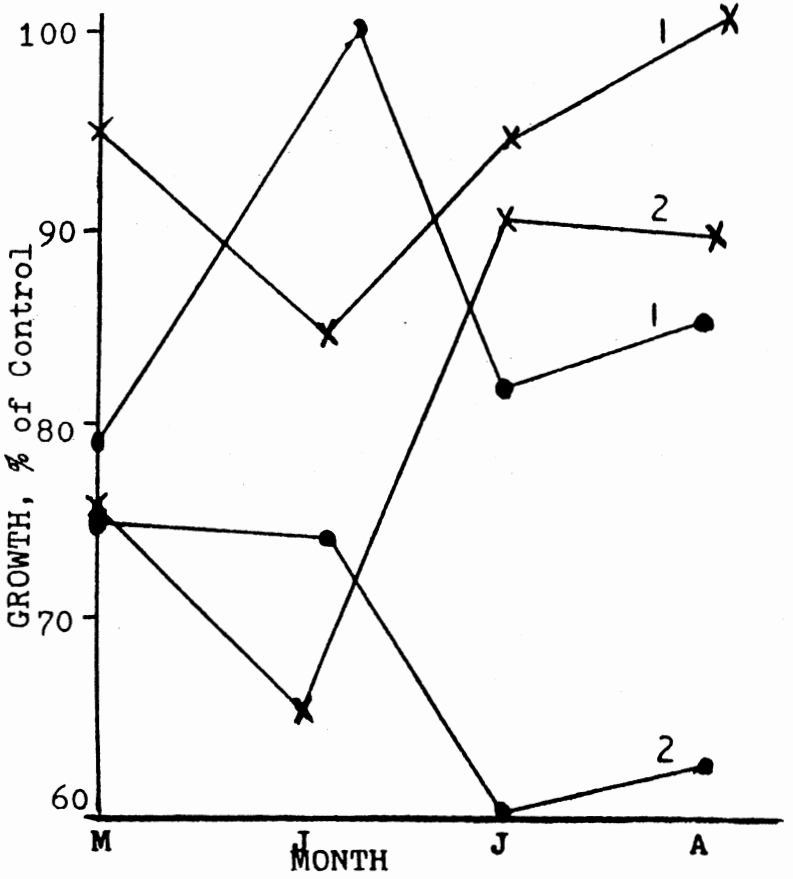


Figure 12. Growth as a Percentage of Control of Wheat (×) and Cheat (•) Shoots Treated with Water Extracts of Ground *Psoralea tenuiflora* Roots at Concentrations of 2 g/100 ml (1) and 4 g/100 ml (2) from May to August 1988.

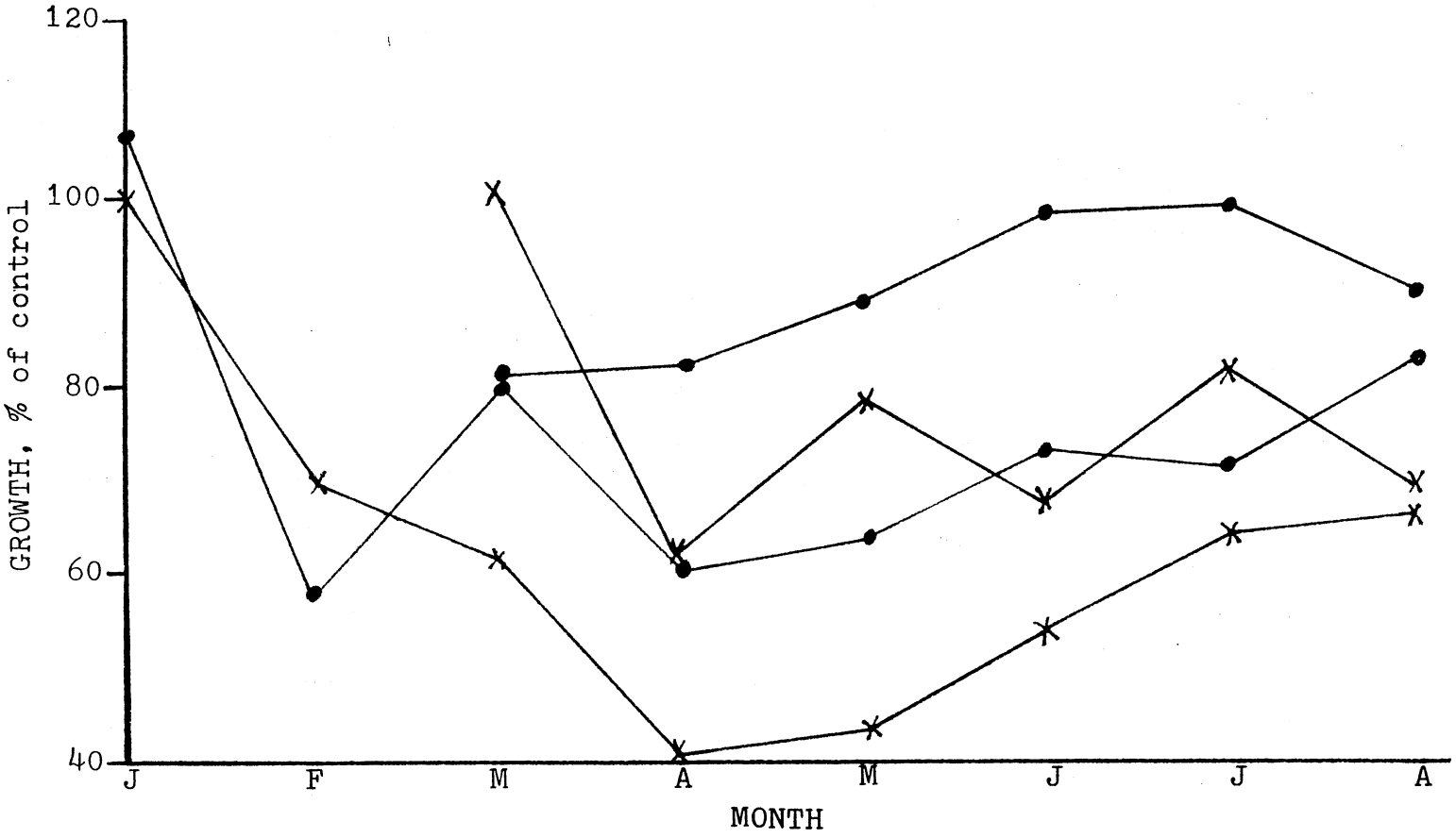


Figure 13. Growth as a Percentage of Control of Wheat (×) and Cheat (•) Shoots Treated with Water Extracts of Ground Advantage Roots at Concentrations of 2 g/100 ml (March-August 1988) and 4 g/100 ml (January-August 1988).

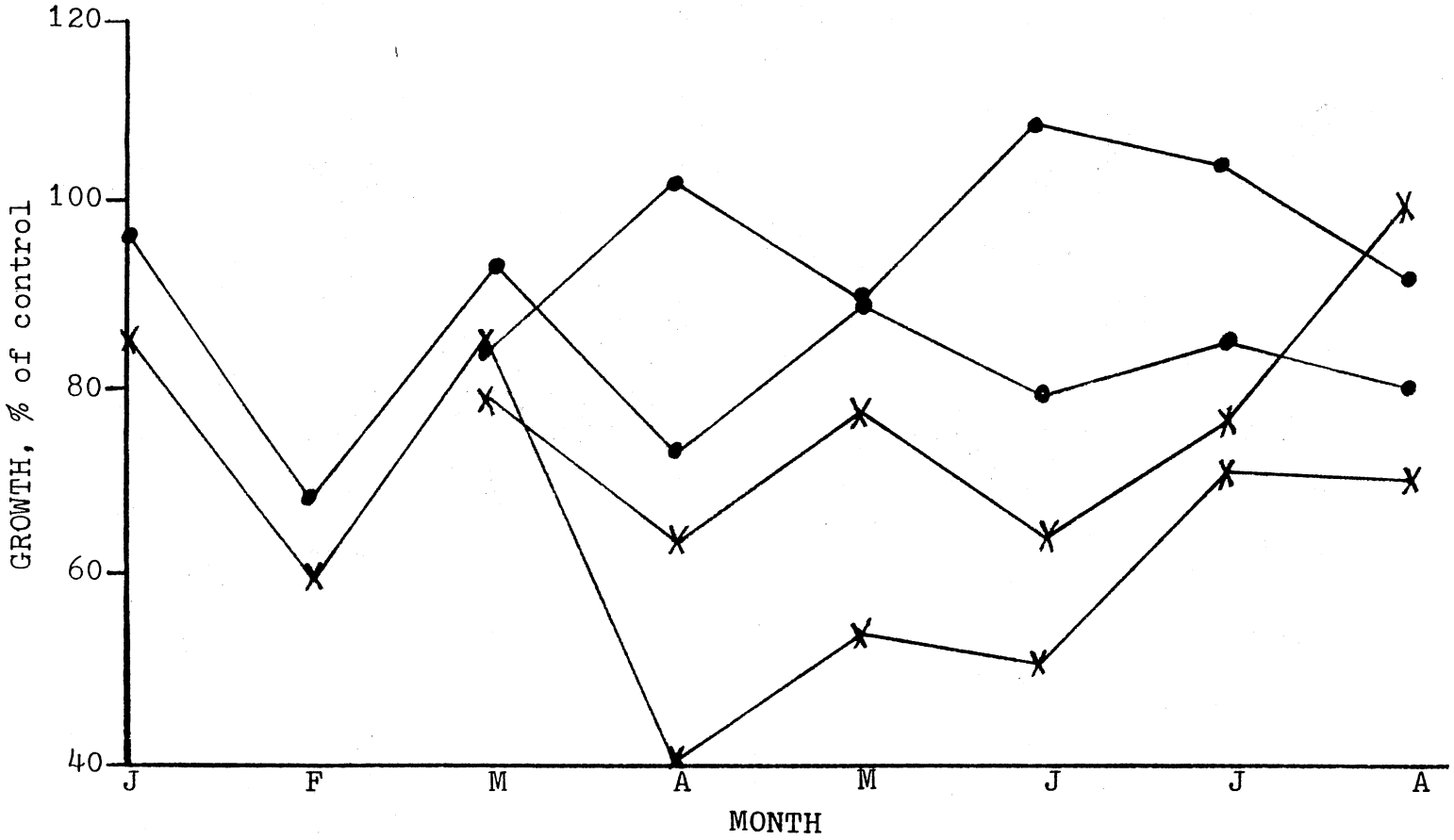


Figure 14. Growth as a Percentage of Control of Wheat (×) and Cheat (•) Shoots Treated with Water Extracts of Ground Baron Roots at Concentrations of 2 g/100 ml (March-August 1988) and 4 g/100 ml (January-August 1988).

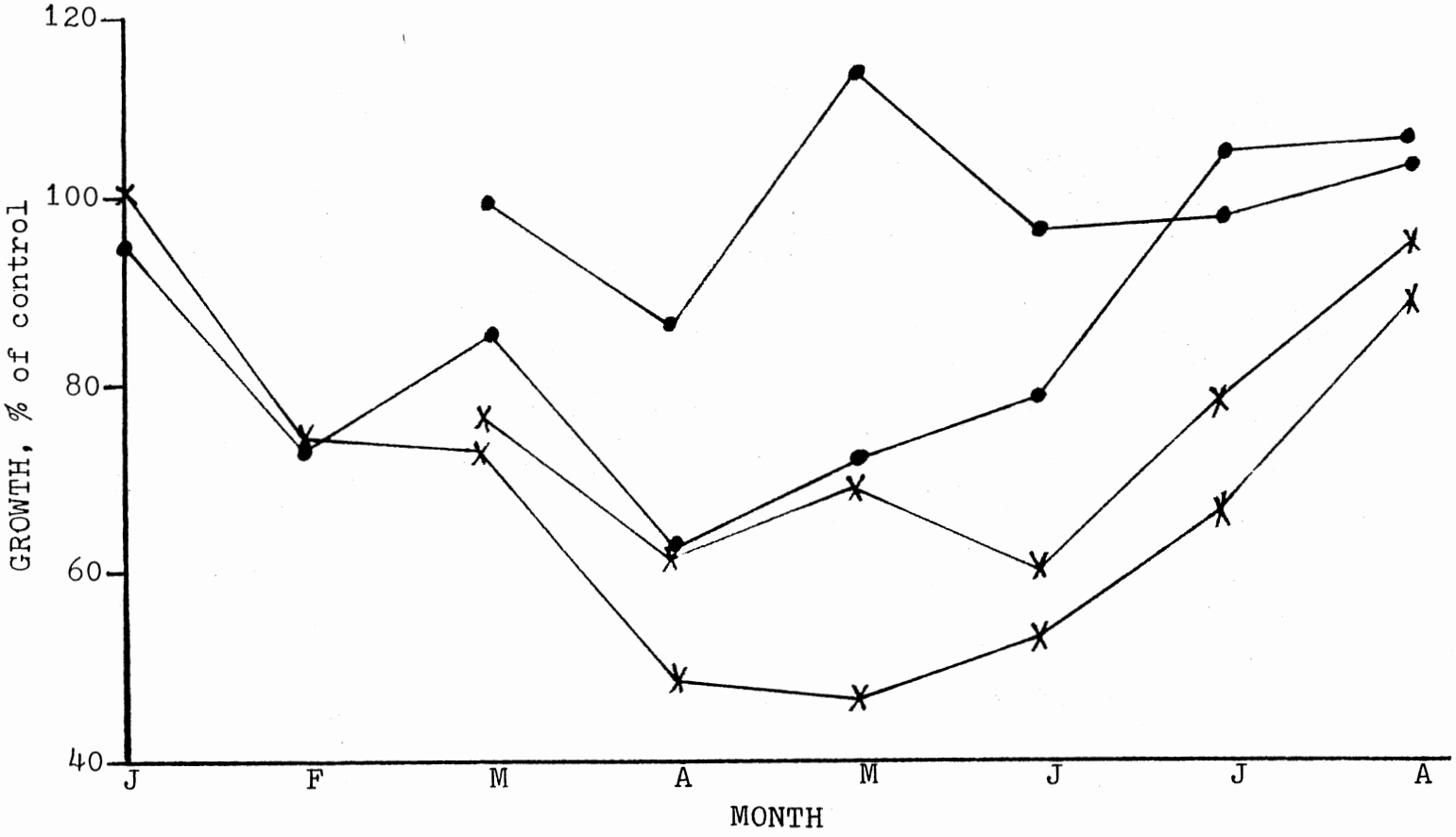


Figure 15. Growth as a Percentage of Control of Wheat (×) and Cheat (•) Shoots Treated with Water Extracts of Ground Cuf 101 Roots at Concentrations of 2 g/100 ml (March-August 1988) and 4 g/100 ml (January-August 1988).

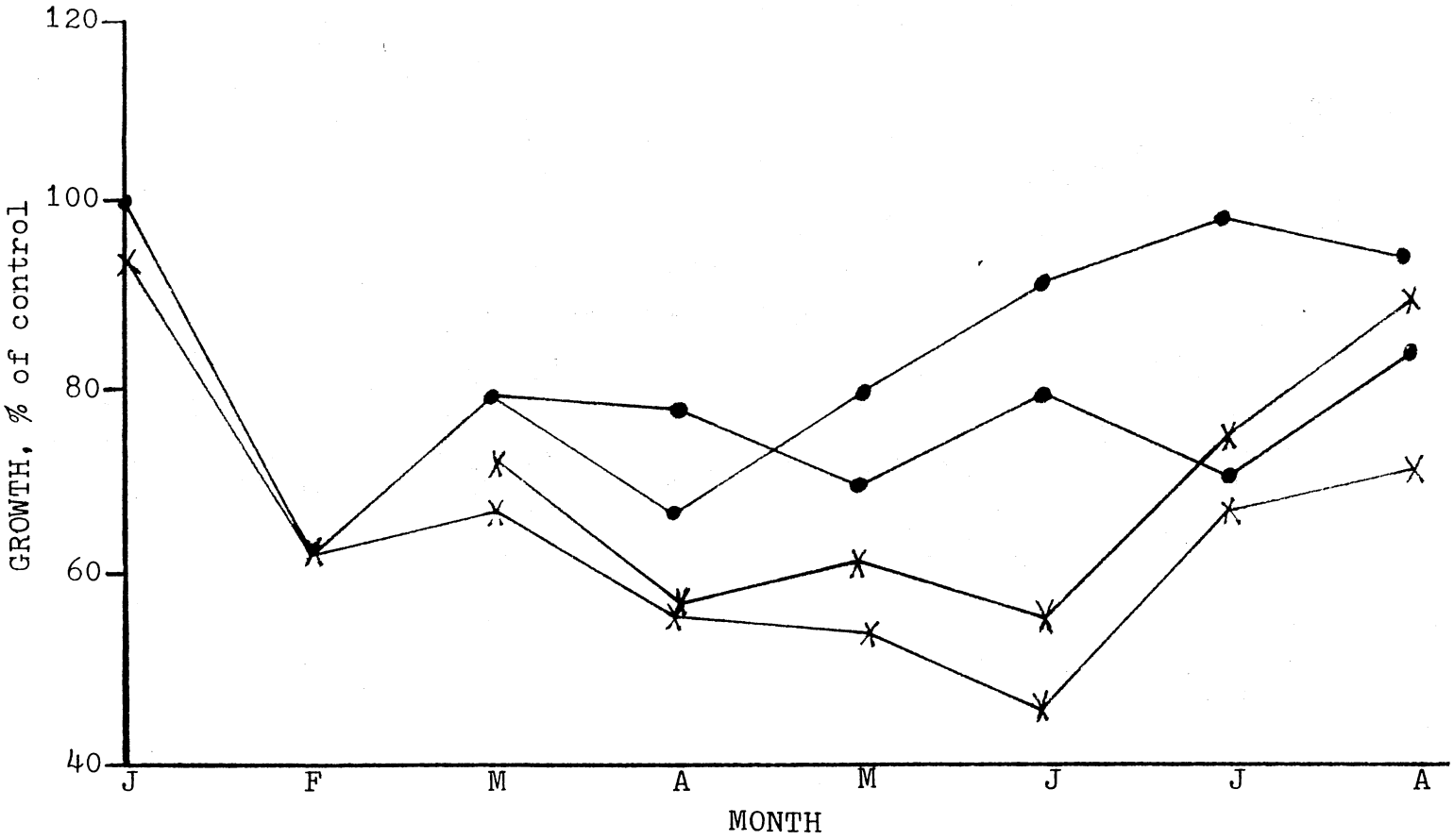


Figure 16. Growth as a Percentage of Control of Wheat (×) and Cheat (•) Shoots Treated with Water Extracts of Ground Granada Roots at Concentrations of 2 g/100 ml (March-August 1988) and 4 g/100 ml (January-August 1988).

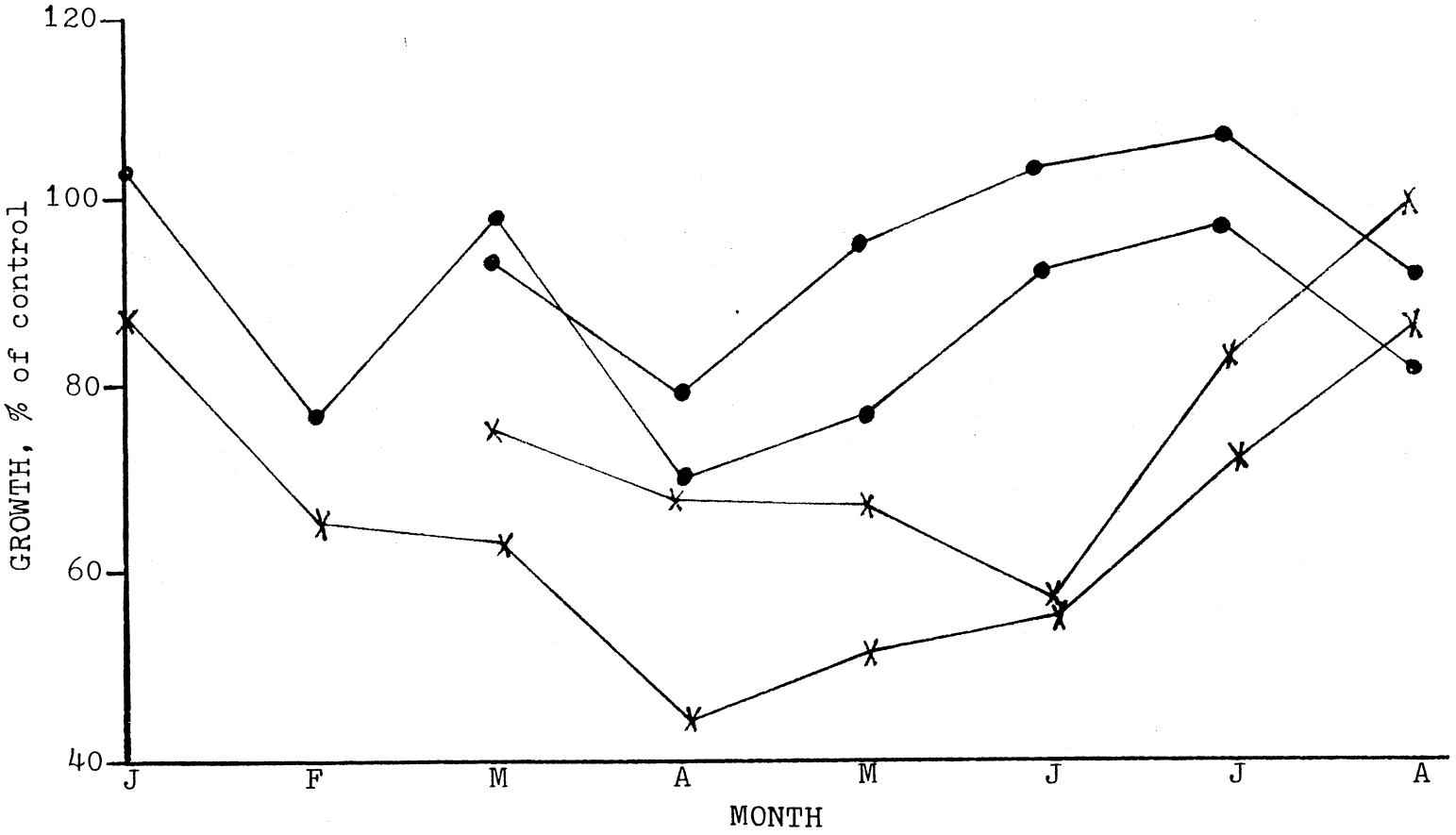


Figure 17. Growth as a Percentage of Control of Wheat (×) and Cheat (•) Shoots Treated with Water Extracts of Ground WL 318 Roots at Concentrations of 2 g/100 ml (March-August 1988) and 4 g/100 ml (January-August 1988).

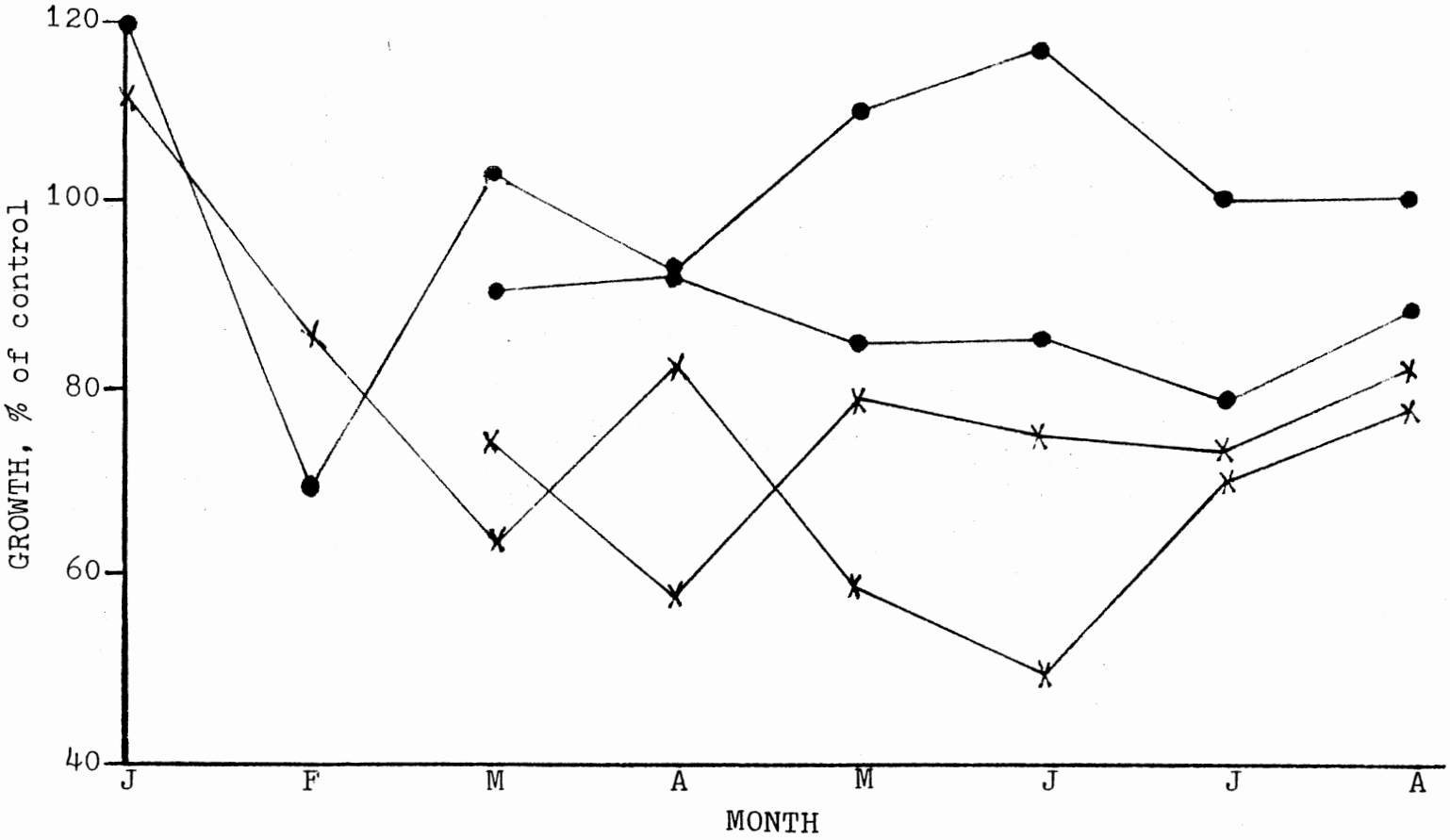


Figure 18. Growth as a Percentage of Control of Wheat (×) and Cheat (•) Shoots Treated with Water Extracts of Ground WL 515 Roots at Concentrations of 2 g/100 ml (March-August 1988) and 4 g/100 ml (January-August 1988).

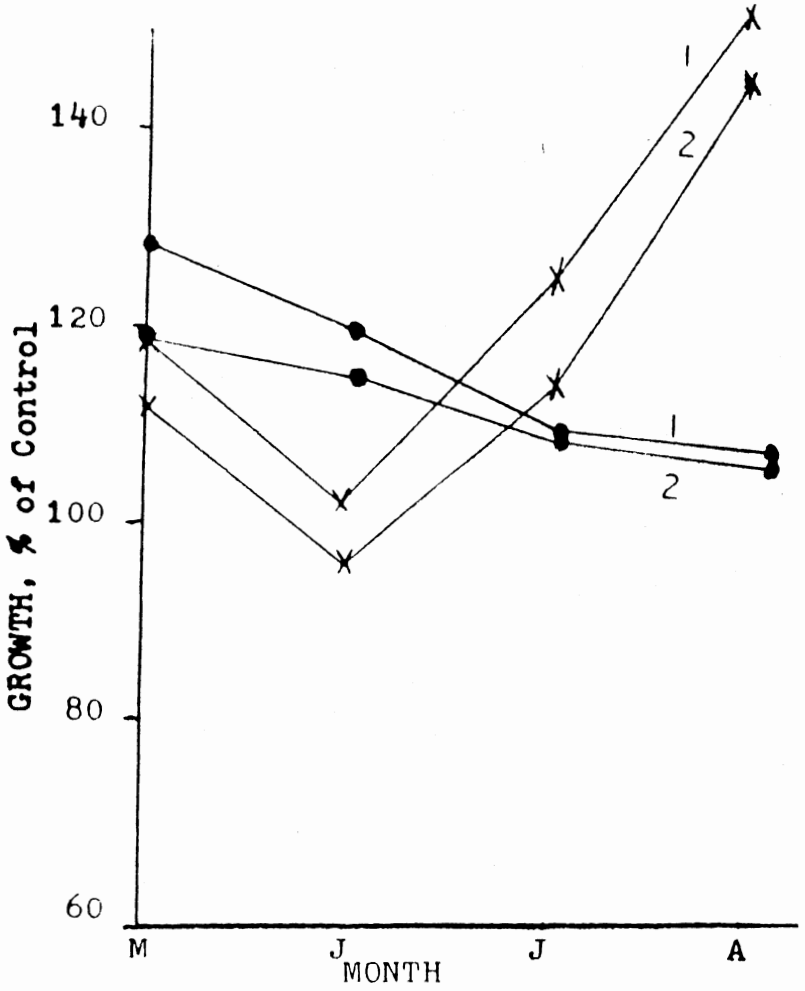


Figure 19. Growth as a Percentage of Control of Wheat (×) and Cheat (•) Shoots Treated with Water Extracts of Ground *L. cuneata* Shoots at Concentrations of 2 g/100 ml (1) and 4 g/100 ml (2) from May to August 1988.

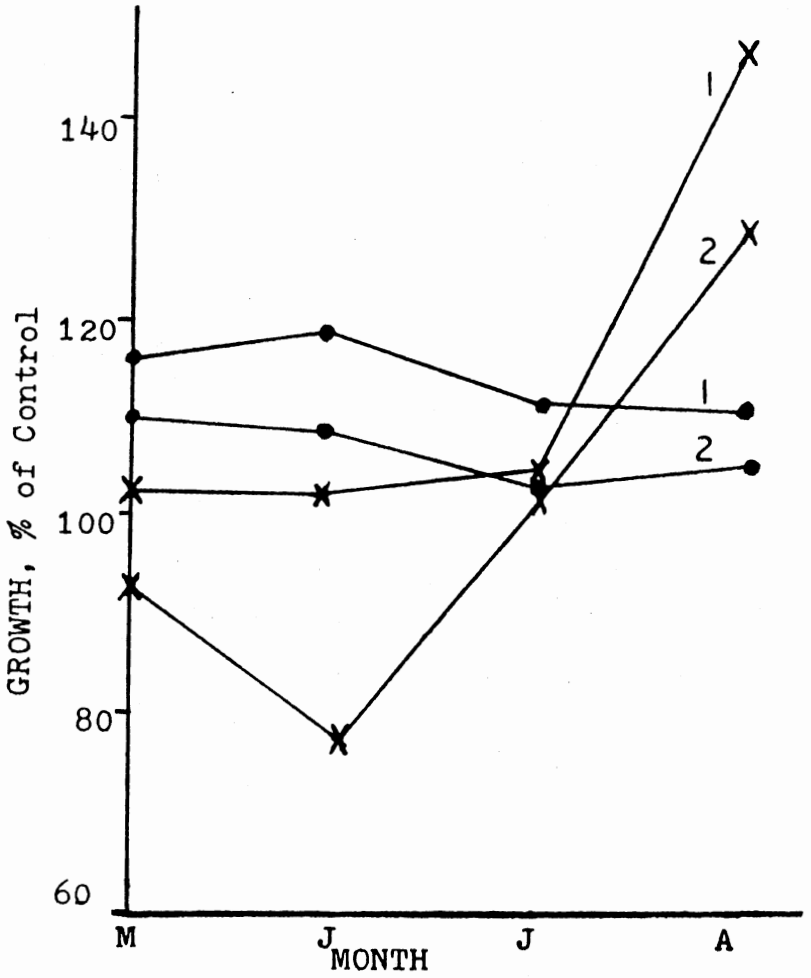


Figure 20. Growth as a Percentage of Control of Wheat (×) and Cheat (•) Shoots Treated with Water Extracts of Ground *P. tenuiflora* Shoots at Concentrations of 2 g/100 ml (1) and 4 g/100 ml (2) from May to August 1988.

growth of wheat and cheat by an average of 21-25% and 26-30%, respectively, and shoot growth was stimulated by nearly all extracts.

The marked variability in the phytotoxicity of the water extracts to wheat and cheat seedling growth with time corresponds to the findings of Hanson *et al.*, Guenzi *et al.*, and Pedersen (1963, 1964, 1975). Guenzi *et al.* found that extracts of alfalfa forage at the bud stage highly inhibited corn seedling root growth, and extracts from alfalfa harvested 25 days after full bloom were least inhibitory. Differences in inhibition obtained from this research correspond to their findings since the highest inhibition recorded during the sampling period was during April, the month in which the first buds appeared in most cultivars, and the lowest inhibition recorded was in June, which is approximately one month after full bloom.

Trichoderma viride Bioassay Results

Statistical Analysis

The results of Student's t-tests comparing *T. viride* growth as affected by water extracts of the six cultivars each month are given in Table VI. Since many more highly significant differences between cultivars were detected with this bioassay as compared to the wheat and cheat bioassays, *T. viride* is clearly more sensitive to differences in saponin content. Significant differences between cultivars were detected in 67% of all the comparisons and 38% of those were highly significant ($P < .001$).

Growth Response of *T. viride*

The growth of *T. viride* in response to extracts at a concentration of 4 g/100 ml of all cultivars during the sampling period is given in Figures 21a and b. Granada extracts were most inhibitory to *T. viride* growth, followed by WL 318, Advantage, Baron, Cuf 101 and WL 515 extracts. The highest inhibition of *T. viride* growth by all cultivars occurred

TABLE VI

RESULTS OF STUDENT'S T-TESTS: MONTHLY COMPARISONS BETWEEN *TRICHODERMA VIRIDE* GROWTH AS AFFECTED BY WATER EXTRACTS OF SIX CULTIVARS OF ALFALFA

	Conc.,	January					February					March					April				
Variety	g/100 ml	B	C	G	3	5	B	C	G	3	5	B	C	G	3	5	B	C	G	3	5
A	8	***	***	***	***	***	**	**	ns	*	ns	***	***	***	***	**	***	**	***	***	***
	4	ns	*	**	ns	ns	ns	ns	***	ns	ns	**	ns	***	***	**	***	ns	***	***	**
B	8		ns	***	***	***		ns	*	*	*		**	***	**	ns		**	***	***	*
	4		ns	*	ns	ns		ns	***	ns	ns		**	***	***	**		***	***	***	ns
C	8			***	***	ns			***	*	***			***	***	***			***	***	*
	4			ns	ns	*			***	ns	ns			***	***	**			***	***	**
G	8				*	***				**	ns				***	***				*	***
	4				*	*				***	***				***	***				***	***
3	8					ns					*					***					***
	4					ns					*					***					***

	Conc.,	May					June					July					August				
Variety	g/100 ml	B	C	G	3	5	B	C	G	3	5	B	C	G	3	5	B	C	G	3	5
A	8	***	***	ns	***	***	ns	**	*	ns	ns	*	**	*	ns	ns	**	**	**	*	***
	4	ns	*	***	***	***	ns	ns	ns	ns	ns	ns	***	**	***	*	***	***	**	**	***
B	8		*	***	ns	ns		*	ns	ns	ns		**	ns	**	**		ns	ns	ns	***
	4		**	***	***	**		ns	ns	ns	ns		**	ns	**	*		ns	*	*	***
C	8			***	**	***			*	**	*			*	***	***			ns	ns	***
	4			***	*	ns			ns	*	*			*	ns	*			*	*	***
G	8				***	***				*	ns				**	**				ns	***
	4				*	***				ns	ns				*	ns				ns	***
3	8					ns					ns					ns					***
	4					*					ns					ns					***

A = Advantage; B = Baron; C = Cuf 101; G = Granada; 3 = WL 318; 5 = WL 515

* = P < .05; ** = P < .01; *** = P < .001; ns = not significant

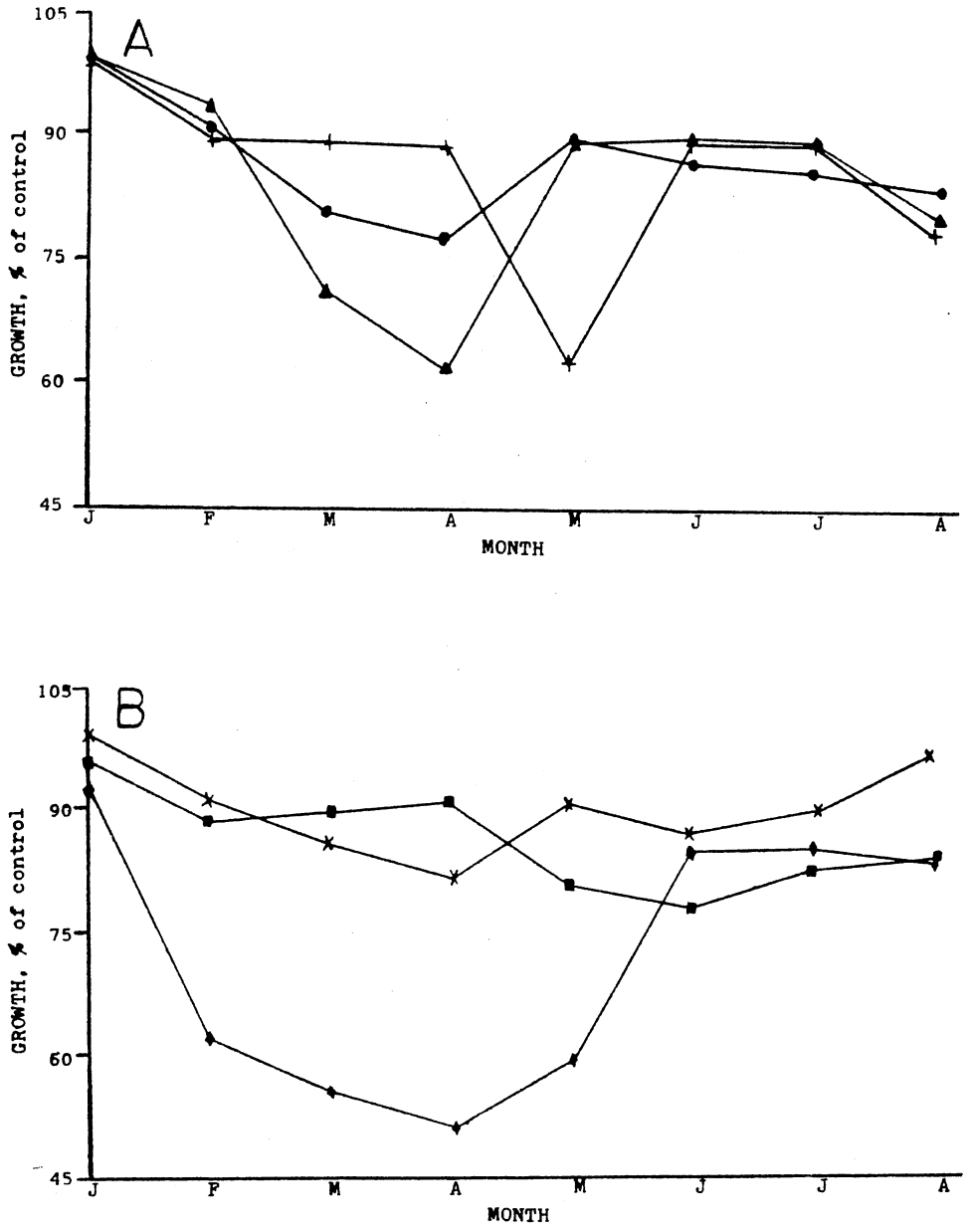


Figure 21. Growth as a Percentage of Control of *Trichoderma viride* Colony Diameters as Influenced by the Water Extracts of a) Advantage (+), Baron (•), WL 318 (▲) and b) WL 515 (■), Granada (◆) and Cuf 101 (×) Roots at a Concentration of 4 g/100 ml from May to August 1988.

in March, April and May, the months with active growth and the highest rainfall. With the exception of Granada, the more dormant cultivars were generally but not consistently more inhibitory to *T. viride*. The data suggest a relationship between the degree of winter dormancy and the extent of fungitoxicity. The growth of *T. viride* in response to the extracts of *L. cuneata* or *P. tenuiflora* was inhibited only slightly or not at all.

Saponin Quantification

A standard curve for the quantification of saponins (Figure 22) was constructed by plotting the growth inhibition caused by the saponin standard at six concentrations ranging from 0.25-3.0 mg saponin per petri dish, which corresponds to the amount of saponin in 33 ml of the potato dextrose agar medium. Only medicagenic acid glycosides were included in the saponin standard, thus only the most biologically active fraction was determined. The biological activity of the other saponins from the different cultivars, soyasapogenol B and hederagenin glycosides, was therefore not determined.

The biologically active saponin content (in mg saponin/g dried roots) of all cultivars and plant species tested during the sampling period is given in Table VII. Granada roots had the highest content, with values ranging from 8.3-37.5 mg biologically active saponin/g roots. The next highest contents were in WL 318 roots, followed by Advantage, Cuf 101, Baron and WL 515 roots. The biologically active saponin content of *L. cuneata* and *P. tenuiflora* dropped sharply from 9.4 and 8.1 mg saponin /g dry roots in May, respectively, to zero in August.

The levels of inhibition of *T. viride* growth reported here, at concentrations of 1 and 2 mg/dish, inhibited growth by 9% and 35%, respectively, and are similar to those found by Jurzysta in his earlier work (1979). This finding should be expected since Jurzysta did not have isolation techniques perfected at the earlier date.

Oleszek and Jurzysta later (1986) reported that medicagenic acid glycosides isolated from *Medicago media* roots were much more biologically active than glycosides of

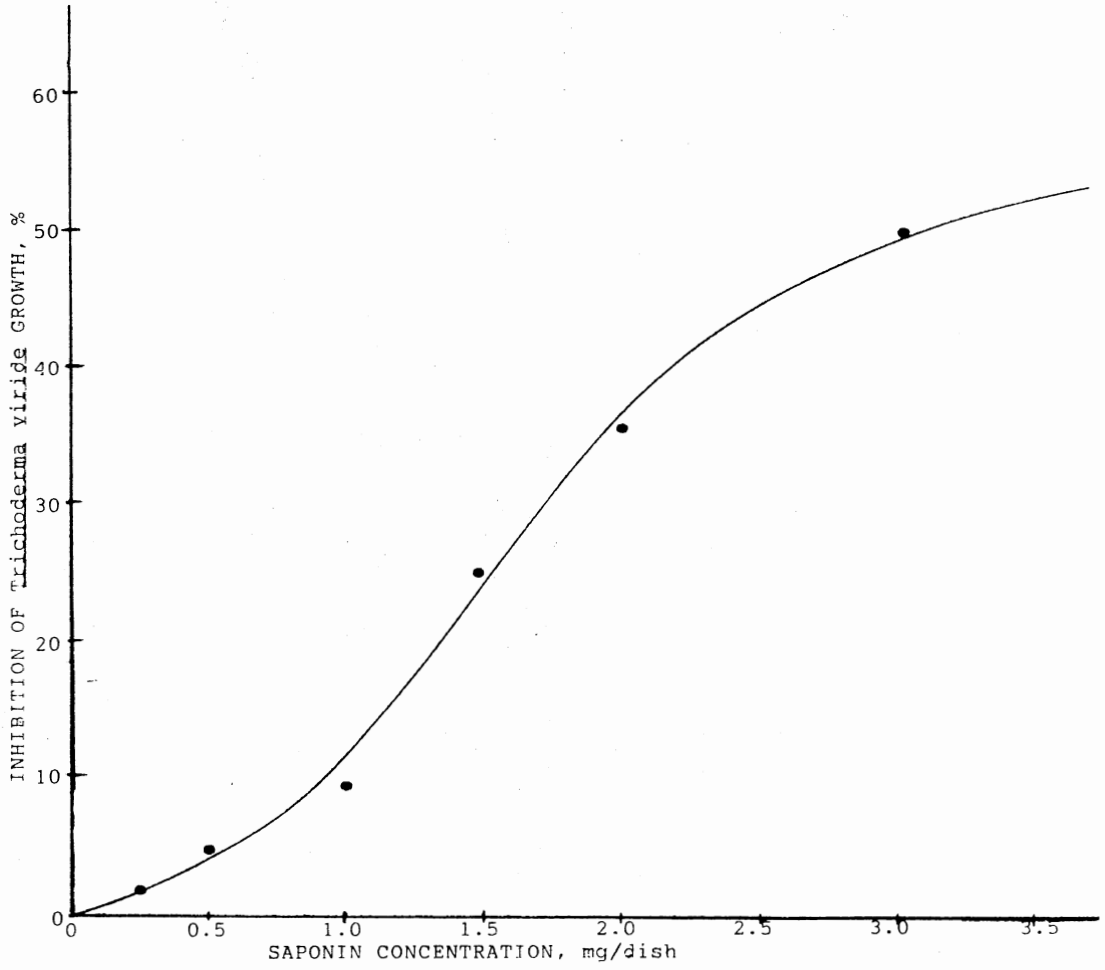


Figure 22. Standard Curve for the Quantification of Biologically Active Saponins.

TABLE VII

BIOLOGICALLY ACTIVE SAPONIN CONTENT OF SIX CULTIVARS OF ALFALFA AND A NATIVE AND A NATURALIZED LEGUME AS DETERMINED FROM THE STANDARD CURVE OF *TRICHODERMA VIRIDE* INHIBITION

	Month							
Source	January	February	March	April	May	June	July	August
Advantage	0.0	10.5	10.5	12.1	28.1	12.1	11.6	17.5
Baron	0.1	10.9	16.3	18.1	11.9	15.0	13.6	15.3
Cuf 101	4.8	12.3	11.3	10.1	16.1	17.4	15.9	15.3
Granada	8.3	23.4	32.1	37.5	27.9	14.5	14.5	15.0
WL 318	0.0	8.3	21.3	26.3	11.4	11.1	11.0	10.8
WL 515	0.0	9.8	13.6	16.0	10.9	12.8	11.9	4.3
L. cuneata	—	—	—	—	9.4	7.6	1.3	0.0
P. tenuiflora	—	—	—	—	8.1	5.9	5.6	0.0

soyasapogenol B and hederagenin. A concentration of medicagenic acid glycosides corresponding to 1 mg/dish inhibited *T. viride* growth by 70%, whereas the same concentration of soyasapogenol B and/or hederagenin glycosides inhibited growth only 8%.

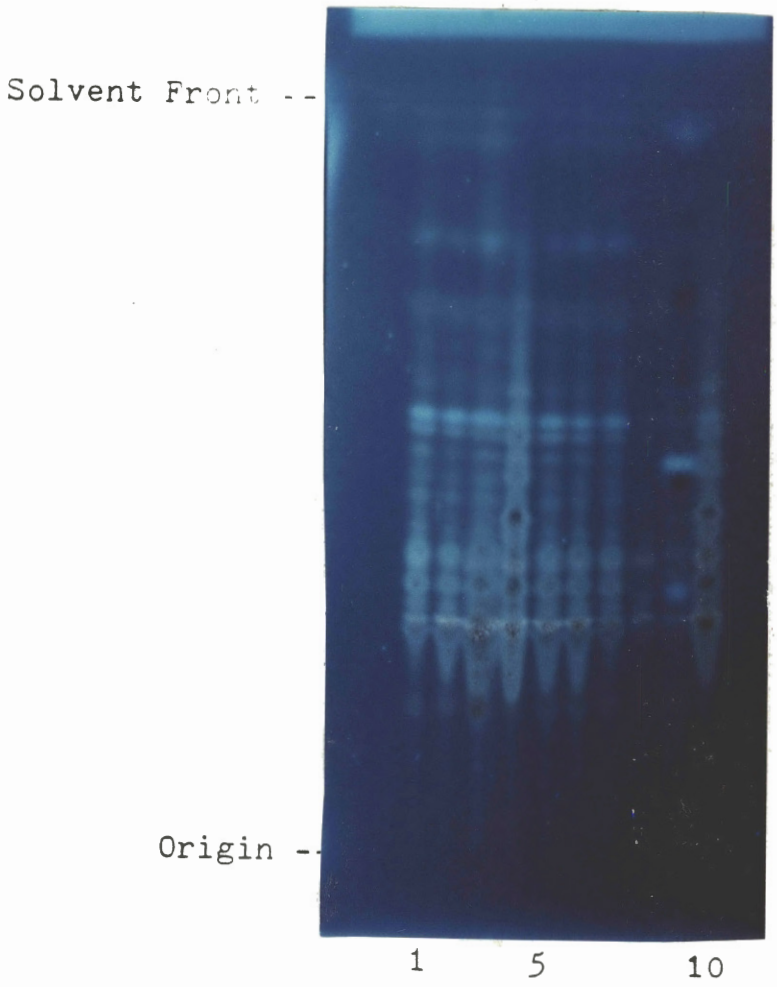
The difference in the response of *T. viride* to medicagenic acid glycosides cannot be readily explained. Perhaps the cultivars from which the standards were isolated differed greatly in the type of medicagenic acid glycosides, which could influence antifungal activity (Oleszek and Jurzysta, 1988). Nonaka (1986) found that different strains of *T. viride* exhibited variable sensitivity to alfalfa saponins, but it is unlikely that this is the case here because the *T. viride* culture came from Dr. Jurzysta's lab.

A qualitative as well as a quantitative difference in the biologically active saponin fraction is suggested by the data. This is consistent with the findings of other researchers (Zimmer *et al.*, 1967; Pedersen *et al.*, 1975). Granada root extracts were not markedly inhibitory to wheat and cheat seedling growth, and statistical analyses of the bioassay results indicate little difference in the saponin content between cultivars. However, Granada, WL 318 and Advantage root extracts are markedly more inhibitory to *T. viride* growth than extracts of the other three cultivars. Differences in the stereochemistry or the water solubility of the saponins may greatly affect their antifungal activity (Scardavi and Elliott, 1967; Oleszek *et al.*, 1988).

Thin Layer Chromatography Results

Isolation of Intact Saponins

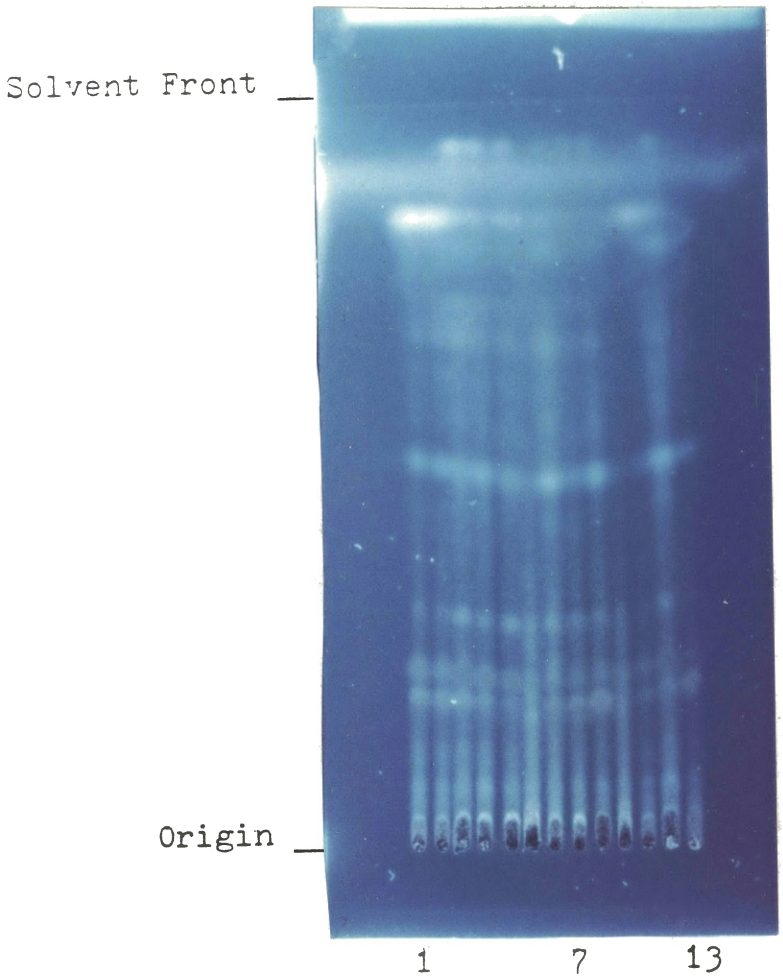
The number of saponins visualized under UV_{366nm} light on the thin layer chromatograms after spraying with the Liebermann-Burchard reagent varied with the solvent systems (SS) used to develop the plates (Figure 23a-c). The separation of saponins was greatest in plates developed in SS I (chloroform:methanol:water 65:38:10).



MAY SAMPLES

1. Advantage	6. WL 318
2. Baron	7. WL 515
3. Cuf 101	8. L. cuneata
4. Standard	9. P. tenuiflora
5. Granada	10. Standard

Figure 23a. Photograph of a Thin Layer Chromatogram of Alfalfa Root Saponins from the May Samples Developed in Chloroform:Methanol:Water 65:38:10 as Seen Under UV Light.



ADVANTAGE

1. January
2. February
3. March
4. April
5. May
6. Standard 0.15 mg
7. June
8. July
9. August
10. Standard 0.1 mg CUF 101
11. January
12. May
13. August

Figure 23b. Photograph of a Thin Layer Chromatogram of Alfalfa Root Saponins from Advantage (January-August 1988) and Cuf 101 (January, May and August 1988) Developed in Chloroform:Methanol 10:1 as Seen Under UV Light.

ADVANTAGE

1. January
2. February
3. March
4. April
5. May
6. Standard 0.15 mg
7. June
8. July
9. August
10. Standard 0.1 mg CUF 101
11. January
12. May
13. August

Figure 23c. Photograph of a Thin Layer Chromatogram of Alfalfa Root Saponins from Advantage (January-August 1988) and Cuf 101 (January, May and August 1988) Developed in Chloroform:Methanol 20:1 as Seen Under UV Light.

Fourteen different saponins (R_f .26-.86) were detected in the standard and the samples consisted of 16-19 components (R_f .03-.99). The color of the spots ranged from fluorescent violet to blue to brown.

The saponin standard was separated into four components when plates were developed in SS II and III (chloroform:methanol 10:1 and 20:1), with an R_f range in SS II of .02-.30 and in SS III of .02-.19. The R_f range of the samples developed in SS II, which contained 8-9 components (spots), was .02-.91 for the six cultivars, .02-.68 for *L. cuneata* and .02-.83 for *P. tenuiflora.* Samples of the six cultivars developed in SS III contained 10-11 spots with an R_f range of .02-.91. *L. cuneata* samples had four spots with an R_f range of .03-.51 and *P. tenuiflora* samples had eight spots with an R_f range of .03-.82.

All samples of the six cultivars developed in the different solvent systems had spots corresponding to the standard saponin components. The intensity of the spots varied from month to month, but the variation in intensity was not great enough to establish any possible correlations between biological activity and the relative abundance of particular compounds.

Since the samples contain more spots than the standard, they probably contain compounds besides saponins. It is likely these other compounds are phenolics (Waller, 1988). The most abundant non-saponin component, present in all six cultivars, had an R_f range of .09-.20 in the various solvent systems and was fluorescent blue under UV light. Phenols are inhibitory to plant germination and growth, so growth inhibition may not be due solely to the saponin fraction of the root extracts. Comparisons between the saponin content of cultivars are still valid since the non-saponin compounds were present in all extracts in approximately the same quantity (as indicated by the intensity of the spot).

Isolation of Aglycones

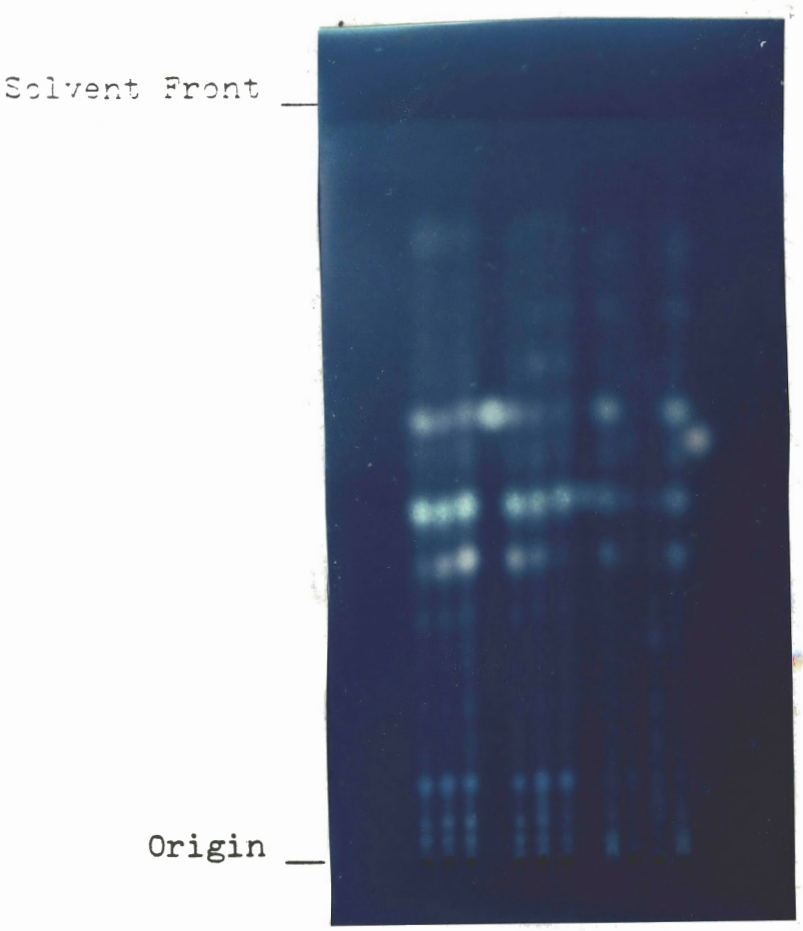
Figures 24a and b contains photographs of the acid-hydrolyzed May samples and standards. The presence of medicagenic acid, soyasapogenol B and hederagenin in all of the cultivars was confirmed. *L. cuneata* and *P. tenuiflora* appeared to contain medicagenic acid and hederagenin, and the latter also contained soyasapogenol B. Medicagenic acid had an R_f value of .42 in SS IV (petroleum ether:chloroform:acetic acid) and .06 in SS V (benzene:methanol 92:8) and the color of the spot was bright pink. The peach-colored hederagenin spot had an R_f value of .59 in SS IV and .16 in SS II, and the yellow soyasapogenol B spot had an R_f value of .56 in SS IV and .22 in SS V. Acid-hydrolyzed samples from the six cultivars contained 14 spots (R_f .01-.86) when developed in SS I and 11 spots (R_f .01-.73) when developed in SS II.

The R_f values obtained for the samples and the standards were similar to those obtained by other researchers (Oleszek and Jurzysta, 1986; Nonaka, 1986). A greater number of spots of the sample relative to the spots of the standard as compared to the intact saponins may be due to the formation of artifacts (i.e. soyasapogenols C-F) in the samples during acid hydrolysis (Oleszek *et al.*, 1988; Prince *et al.*, 1986).

Capillary Gas Chromatography Results

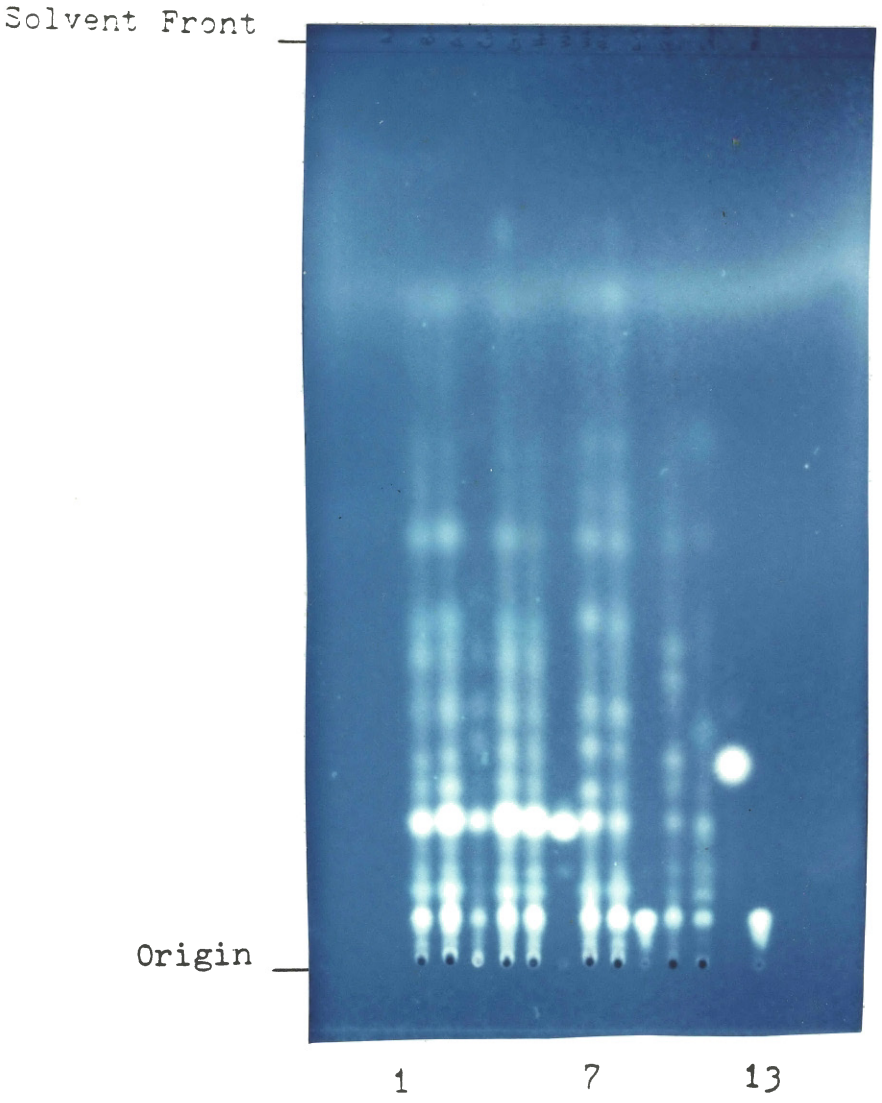
Presence of Aglycones

The presence of medicagenic acid, hederagenin and soyasapogenol B in the derivatized samples of the May samples, as indicated on the thin layer chromatograms, was confirmed. Medicagenic acid and soyasapogenol B were also present in *L. cuneata* and *P. tenuiflora* samples, and hederagenin was present in the latter. The retention times, relative peak area and concentrations of aglycones and standards from alfalfa root saponins are given in Table VIII. Peaks were assigned a letter based on their retention time (RT).



1. Advantage
2. Baron
3. Cuf 101
4. Hederagenin
5. Granada
6. WL 318
7. WL 515
8. Medicagenic acid
9. Standard
10. P. tenuiflora
11. L. cuneata
12. Standard
13. Soyasapogenol B

Figure 24a. Photograph of a Thin Layer Chromatogram of Alfalfa Root Aglycones from the Acid-Hydrolyzed May Samples and Standards Developed in Petroleum Ether:Chloroform:Acetic Acid 7:2:1 as Seen Under UV Lights.



1. Advantage
2. Baron
3. Cuf 101
4. Hederagenin
5. Granada
6. WL 318
7. WL 515
8. Medicagenic acid
9. Standard
10. P. tenuiflora
11. L. cuneata
12. Standard
13. Soyasapogenol B

Figure 24b. Photograph of a Thin Layer Chromatogram of Alfalfa Root Aglycones from the Acid-Hydrolyzed May Samples and Standards Developed in Benzene:Methanol 92:8 as Seen Under UV Lights.

TABLE VIII

RETENTION TIMES, RELATIVE PEAK AREA AND CONCENTRATIONS OF AGLYCONES AND STANDARDS FROM ALFALFA ROOT SAPONINS: MAY 1988

Compound	Med. acid[1]	Hederagenin[1]	Soy. B[1]	Granada	Cuf 101	Baron	Advantage	WL 515	WL 318	Standard[2]	P.teniuflora	L. cuneata
A				20.22**	20.08*							20.14**
B				20.77*		20.60*						
C				20.90**		20.77*						
D				21.09*								
E				21.33**							21.39**	
F		21.74***		21.76**		21.79**		21.60***				
G				21.96***	21.93**		21.92**	21.98**	21.98*	21.98*		
H				22.36*								22.11*****
I				22.48*								
J				22.76*			22.99*					
K				23.27*	23.02*	23.13*	23.44**		23.40*			
L			23.84***	23.48***	23.96***		23.82***	23.71***	23.90**	24.00**	23.96**	23.90**
M		24.27****		24.20*	24.28*							
N				24.82*	24.72*	24.99**		24.78*	24.76**			24.86*
O			25.38*****	25.21*	25.30**		25.29**				25.43*	25.39**
P				25.75*		26.48**						
Q				26.33*	26.42**	27.31**	26.37**	26.27**	26.29**		26.50**	
R	27.55***			26.68*	27.96**	27.98**	27.93**	27.92**	27.92**	27.39*****	27.93**	27.32*****
S	28.78*****			28.59*****	28.72*****	28.70*****	28.65*****	28.62*****	28.64*****	28.67***	28.63***	28.60***
T[3]				29.55***	29.71***	29.60***	29.49***	29.52**	29.52***		29.50*	
U					30.04**			30.11**	30.16**			30.20**
V					32.20*		32.00*	30.99*	31.02*			
W				32.98***	33.16***	33.07**			32.98*	33.31**		
X					35.13*				34.94*			
Y				35.46*	35.62*							
Z					37.28*						37.20**	

***** = 50-70% of total peak area of peaks with RT 20 min
**** = 30-50%
*** = 10-30%
** = 1-10%
* = trace

[1] Standards supplied by Dr. M. Jurzysta

[2] Alfalfa root saponins isolated from Cimarron cultivar

[3] Considered to be an isomer of medicagenic acid

Only peaks with a RT of more than 20 min were examined because the earlier peaks were considered byproducts of the methylation and trimethylsilylation reactions.

The gas chromatograms of the May 1988 samples analyzed are located in Appendix A, Figures 25a-l. Samples from the six cultivars contained 10-22 peaks. Granada extracts contained the highest number and WL 515 extracts contained the lowest number. The alfalfa root saponin standard sample had five peaks and the aglycone standards had two peaks each.

Retention times of the peaks corresponding to S, the largest medicagenic acid standard peak ranged from 28.59-28.78. This peak constituted 50-70% of the total peak area in the six cultivars and 10-30% of the peak area in *L. cuneata* and *P. tenuiflora*. Peaks corresponding to soyasapogenol B constituted 10-30% of the total peak area in all cultivars except WL 318. The area of peaks corresponding to hederagenin varied among the samples from 0-30% of the total peak area.

Quantification of the Aglycones

Since a known amount (20 ng) of the aglycone standards were injected, the area of the standard peak can be compared to the area of the peaks from the samples and quantities of the aglycones in the samples can be determined. These calculated quantities expressed as mg aglycone/g dried roots, are given in Table IX. Medicagenic acid is present in quantities at least six times greater than the other aglycones in all samples. This agrees with the findings of other researchers (Jurzysta and Jurzysta, 1978; Nonaka, 1986; Oleszek *et al.*, 1988) that medicagenic acid glycosides predominate the alfalfa root saponin fraction. Peaks believed to be isomers of medicagenic acid were included in the calculations. This is probably the first time isomers of medicagenic acid have been reported. The peaks, which were approximately the same size and shape, appeared directly after the large medicagenic acid peak in all cultivars.

TABLE IX

QUANTITIES OF MEDICAGENIC ACID, HEDERAGENIN AND SOYASAPOGENOL B IN THE MAY SAMPLES AS DETERMINED BY PEAK AREA

	Quantity, mg/g roots							
Aglycone	Adv.	Bar.	Cuf	Gra.	318	515	*L. c.*	*P. t.*
Med. acid[1]	1.32	1.28	1.17	1.11	0.91	1.03	0.11	0.17
Soy. B[2]	0.23	0.11	0.20	0.10	0.01	0.15	0.04	0.00
Hed.[3]	0.22	0.01	0.06	0.35	0.03	0.54	0.06	0.01

[1]Includes peaks R and S and T, believed to be an isomer of medicagenic acid.
[2]Includes peaks L and O.
[3]Includes peaks F and M.

The amount of the aglycones did not appear to be correlated with the extent of winter dormancy or the biological activity of the aqueous extracts of the six cultivars. This suggests that the biological activity may be dependent on the structure of the saponins rather than on the amount and type of aglycones present (Pedersen, 1967; Nonaka, 1986; Oleszek *et al.*, 1988). In order for conclusive evidence of any correlations to be established, analysis of the other monthly samples would be required.

This is the first known publication of capillary gas chromatographic data on alfalfa root saponins. The retention times of the aglycone standards relative to each other corresponds to the findings of Brawn *et al.* (1981) and Jurzysta and Jurzysta (1978), who used packed columns. The quantification data may not represent the entire amount of saponins in the roots since Brawn *et al.* reported that medicagenic acid was still being liberated 90 h after the onset of acid hydrolysis. The samples were hydrolyzed for only four hours, so it is unlikely that the entire aglycone fraction was obtained.

CHAPTER V

SUMMARY AND CONCLUSIONS

The results of this research indicate that the quantity and biological activity of alfalfa root saponins varied with respect to time. The biologically active saponin contents of alfalfa roots as measured by bioassays were generally highest during periods of rapid growth and high rainfall.

The trend of decreased growth of *T. viride* with higher amounts of rainfall may be due to a grater amount of dissolved saponins. During months with low rainfall, saponins may be adsorbed onto soil particles and therefore biologically less active. Another possibility is that alfalfa roots may not produce as many saponins and existing saponins may have been hydrolyzed by enzymes produced within the plant or by soil microorganisms. The biological activity of the saponins may also be due to structural differences. The number of sugar groups attached to the aglycone affects the water solubility of the saponin and they may be attached at different carbon atom positions; thus altering the biological activity of the molecule. Oleszek *et al.* (1988) reported that free substituted groups attached to C-3 (Ring A) or C-28 (Ring E) (Figure 2) were important in determining the extent of biological activity.

Wheat and cheat seedlings were less sensitive to differences in saponin content between cultivars than *T. viride*. Consistent differences between cultivars were not detected in wheat and cheat bioassays, whereas the more sensitive *T. viride* bioassays indicated many highly significant differences between cultivars. Cheat seedling roots were inhibited 8-10% more than wheat seedling roots. The growth of wheat, cheat and *T.*

viride was inhibited considerably less by extracts of *L. cuneata* and *P. tenuiflora* roots than by extracts of the roots of the six cultivars.

An average of 14 different saponins per cultivar were separated by thin layer chromatography. Approximately 20% of the saponins from *L. cuneata* and *P. tenuiflora* had the same R_f as saponins from the alfalfa root standard. The saponins probably differed with respect to the aglycone moiety and the sugar chain length, type and location. The aglycones medicagenic acid, hederagenin and soyasapogenol B were identified in all cultivars by thin layer and capillary gas chromatography. Medicagenic acid and hederagenin were present in lesser amounts in the *L. cuneata* and *P. tenuiflora* extracts.

Medicagenic acid constituted about 50-70%, and hederagenin and soyasapogenol B 10-15% of the alfalfa root saponin fraction. Peaks not identified as the above mentioned aglycones constituted the remainder of the fraction. Soyasapogenol C and F were probably present as they arise from the acid hydrolysis of soyasapogenol B in HCl and water (Jurzysta, personal comunication). Other compounds present may have been unidentified aglycones, phenolic compounds or byproducts of derivatization.

The relative quantities of the aglycones present in the root extracts may be related to the biological activity. Structural differences of constituent saponins may be more important determinants of biological activity. No definite conclusions can be drawn based on the data obtained from only one month's samples, but it seems that the quantities of the aglycones present in the acid-hydrolyzed samples of the six cultivars were not related to the biological activity.

Granato (1983), using high performance liquid chromatography, found that amounts of saponins and other compounds present in soybean roots varied greatly with the stage of growth; he also found no significant differences in the amounts and types of compounds between five soybean cultivars. It is likely that the amount and type of compounds present in the alfalfa roots varies with time, as shown in this study.

Hanson *et al.* (1963) and Oleszek *et al.* (1988) believe that environmental factors influence the production of saponins. Cates (1984) reported that the production of most secondary metabolites increases under environmental stress. To measure the relationship between the environmental parameters and growth inhibition of wheat and cheat seedlings, plots would need to be established in areas with different rainfall and temperature regimes; also other measurements such as soil temperature, moisture and pH would be needed.

Alfalfa root saponins are highly allelopathic compounds. The potential exists to breed crops for higher saponin content that would then suppress weeds. The lack of evidence of a relationship btween the biological activity of the alfalfa root saponins and the amount and type of aglycones in the samples leads to the conclusion reached by other researchers that biological activity is highly dependent on the structure of the saponins. Little work has been done on isolation of the individual saponins from alfalfa roots (Oleszek and Jurzysta, 1988). Further isolation, purification and characterization and elucidation of alfalfa root saponins is suggested before the specific biological activity of certain saponins can be utilized to the benefit of man.

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APPENDIX A

CAPILLARY GAS CHROMATOGRAMS OF ACID-HYDROLYZED, DERIVATIZED MAY SAMPLES AND AGLYCONE STANDARDS

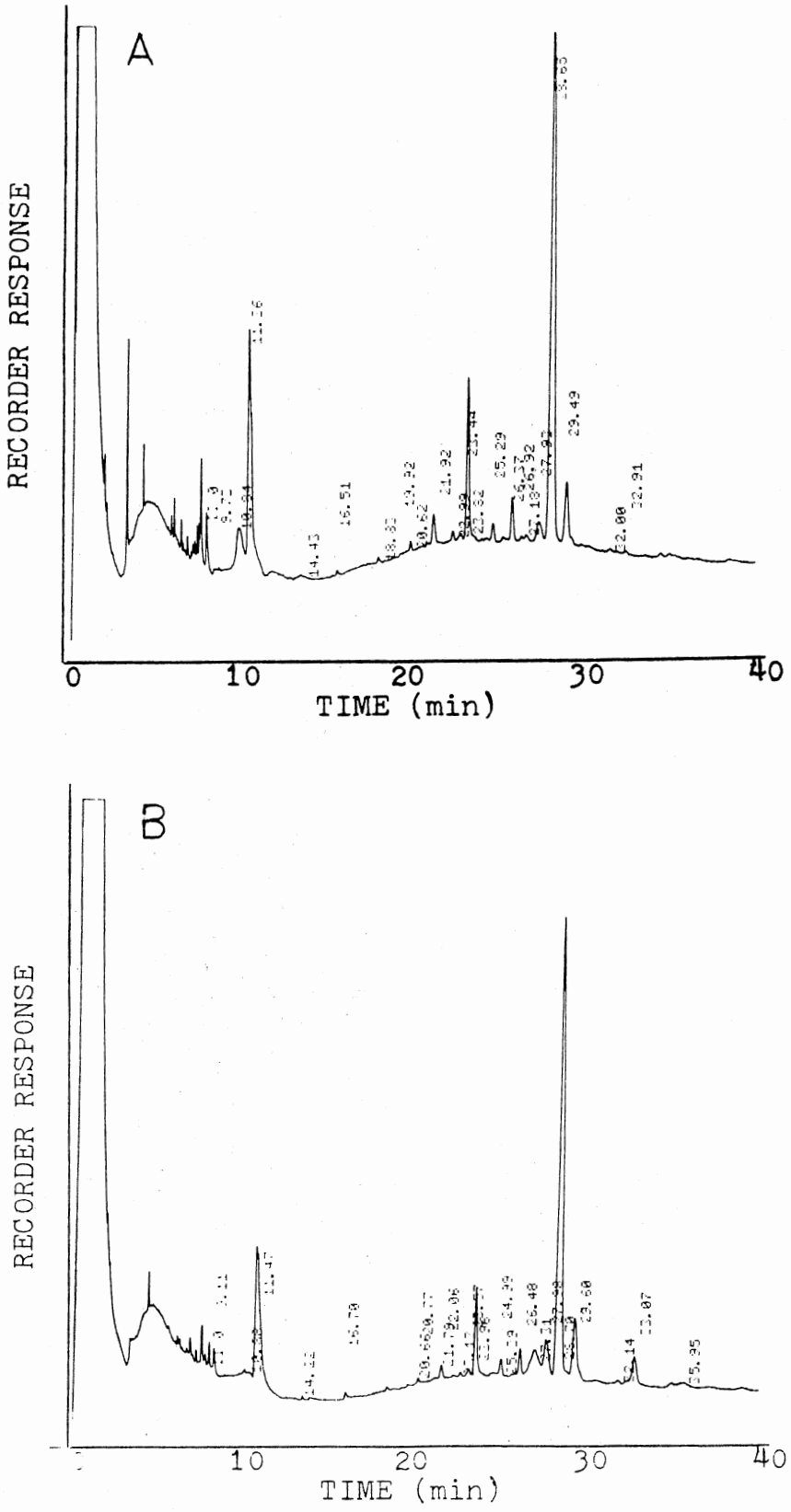


Figure 25. a) Capillary Gas Chromatogram of Advantage Root Extracts.
b) Capillary Gas Chromatogram of Baron Root Extracts.

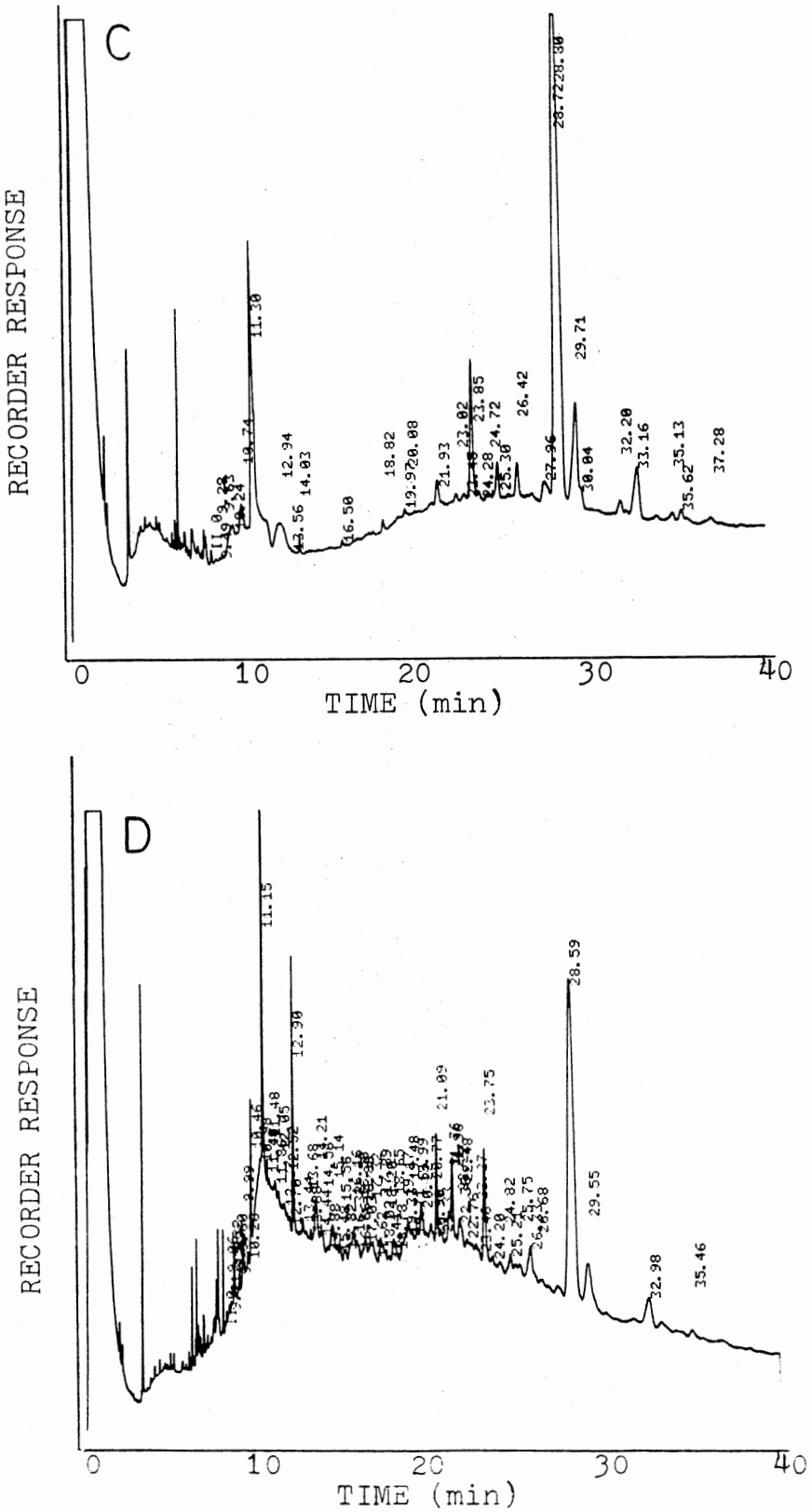


Figure 25. c) Capillary Gas Chromatogram of Cuf 101 Root Extracts.
d) Capillary Gas Chromatogram of Granada Root Extracts.

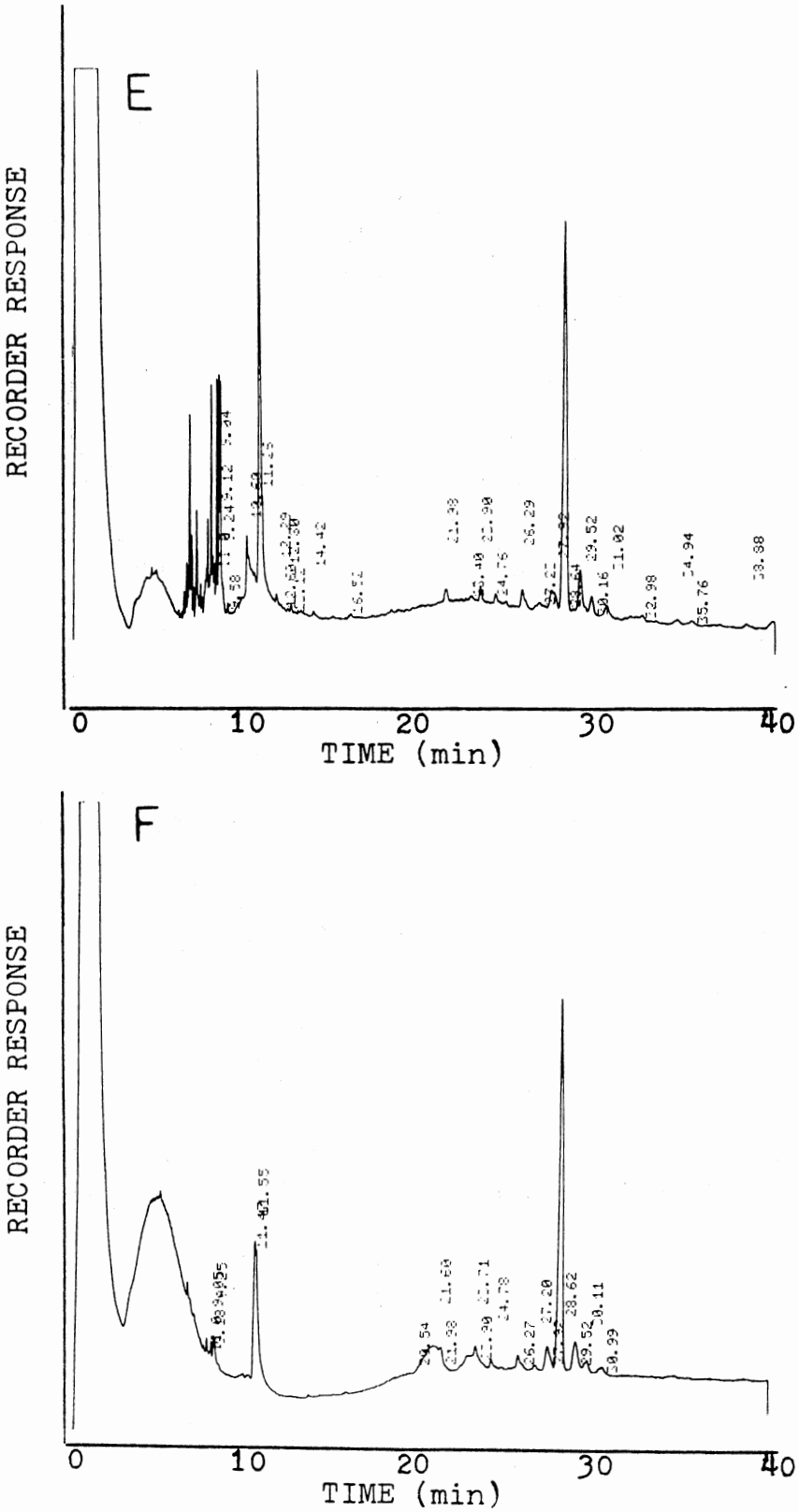


Figure 25. e) Capillary Gas Chromatogram of WL 318 Root Extracts.
f) Capillary Gas Chromatogram of WL 515 Root Extracts.

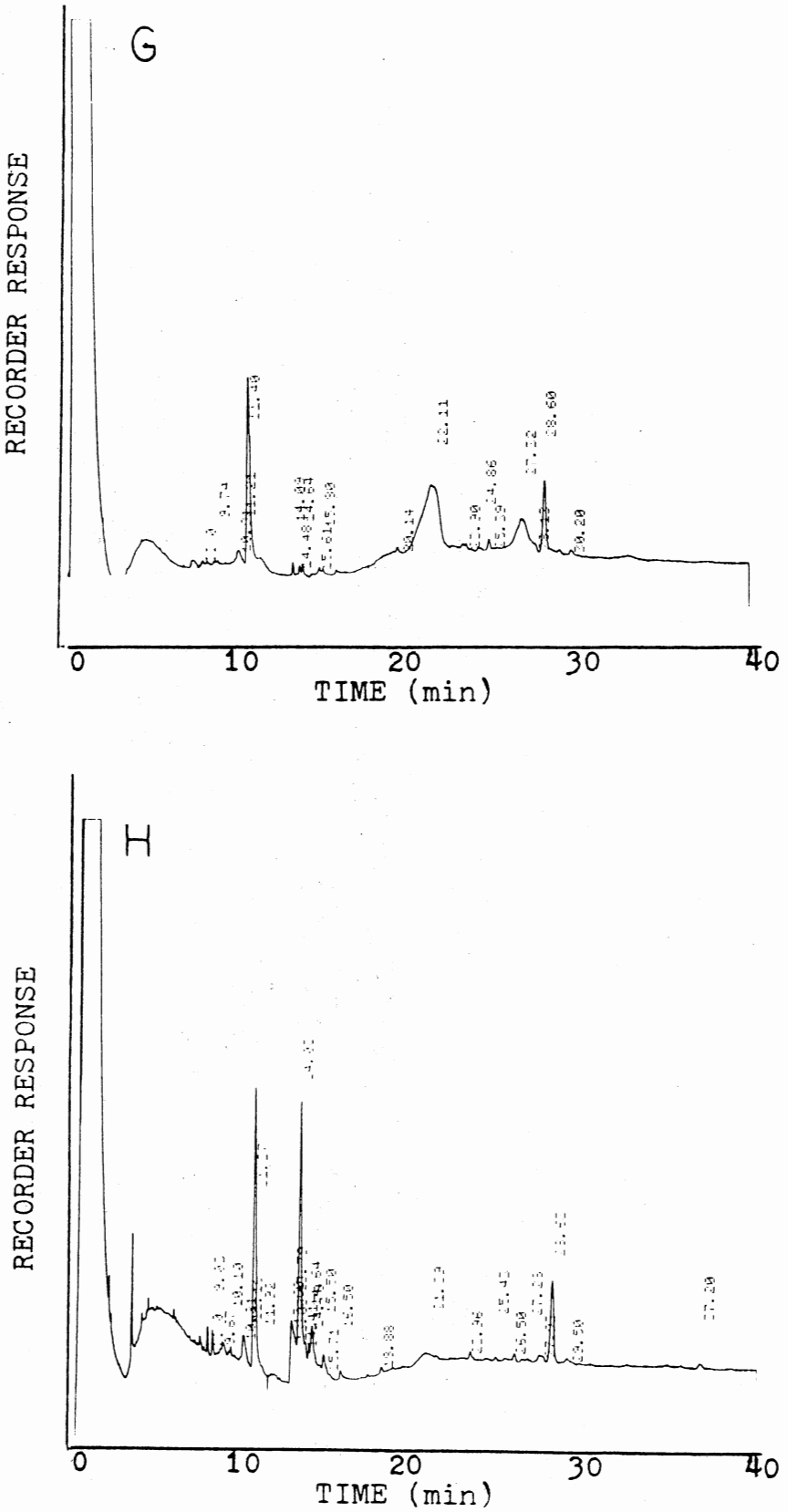
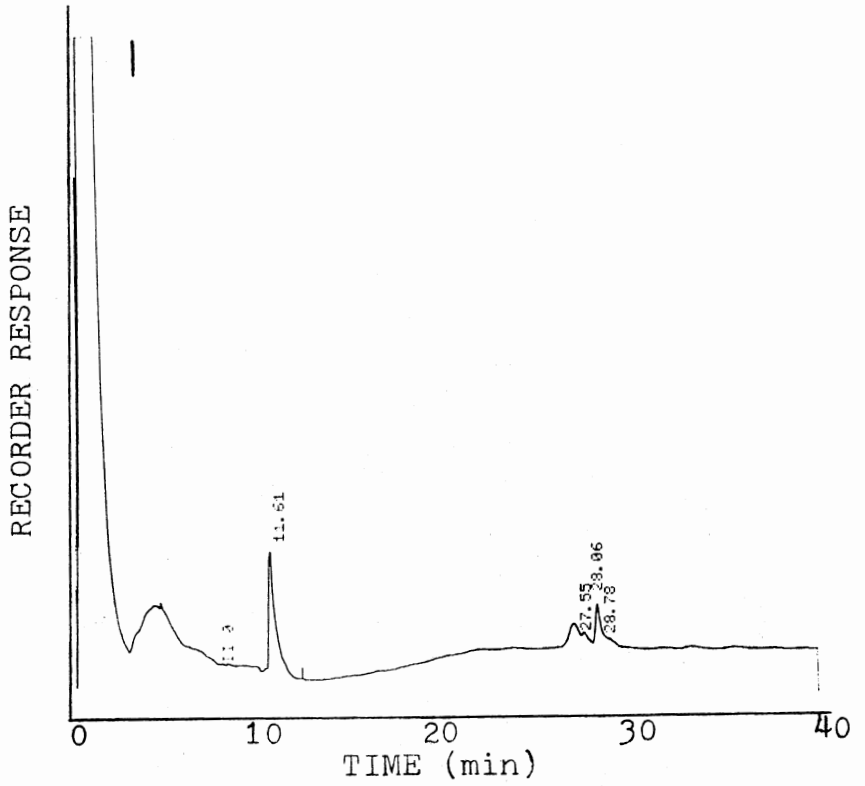


Figure 25. g) Capillary Gas Chromatogram of *L. cuneata* Root Extracts.
h) Capillary Gas Chromatogram of *P. tenuflora* Root Extracts.



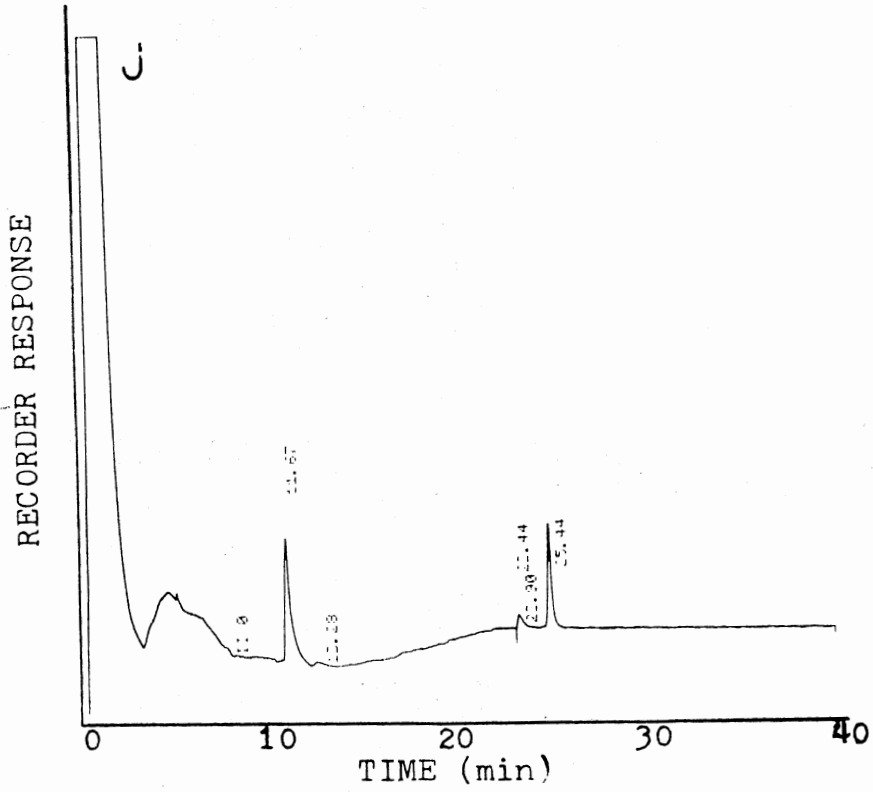


Figure 25. i) Capillary Gas Chromatogram of Medicagenic Acid (20 ng).
j) Capillary Gas Chromatogram of Soyasapogenol B (20 ng).

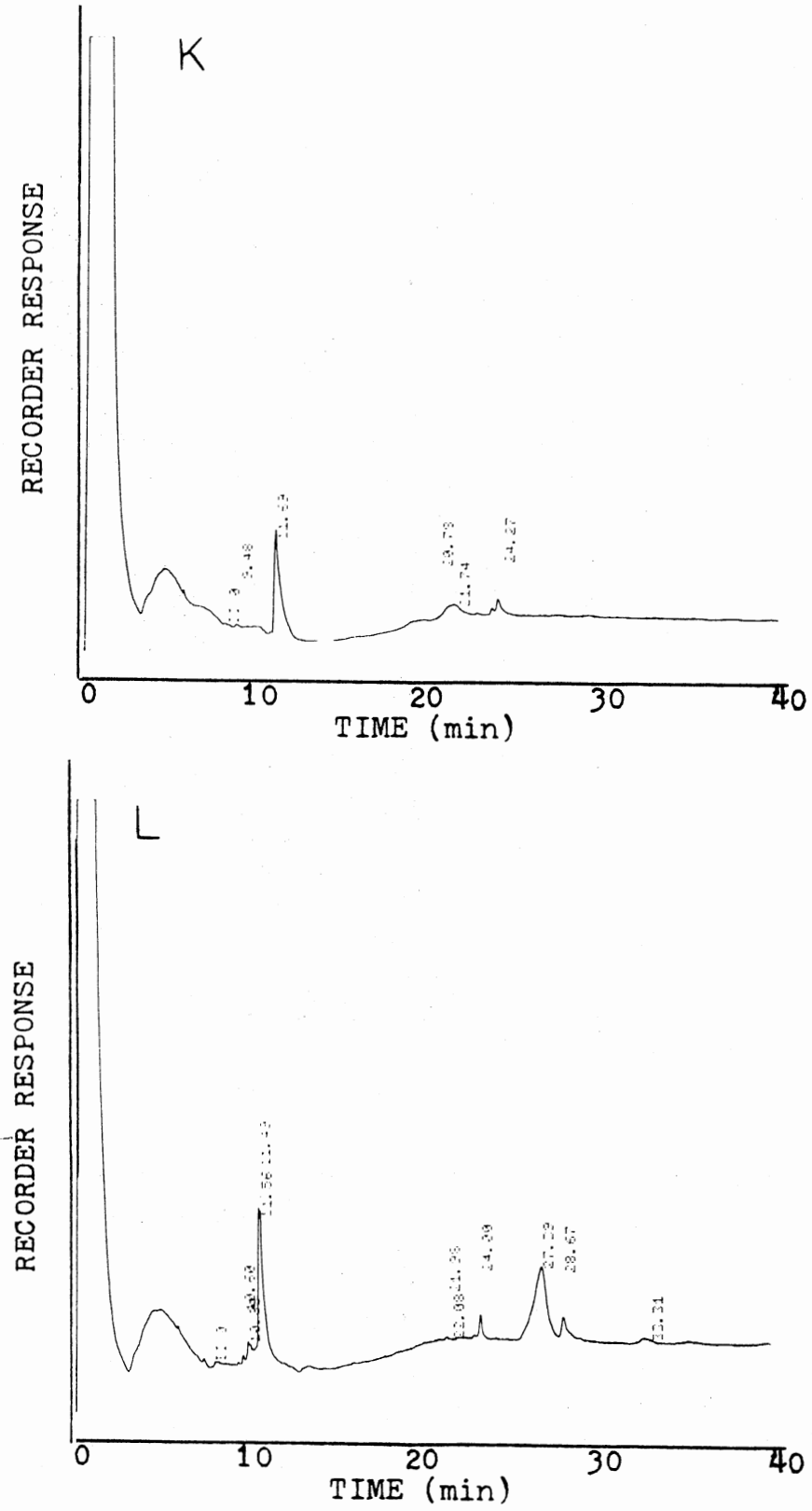


Figure 25. k) Capillary Gas Chromatogram of Hederagenin (20 ng).
l) Capillary Gas Chromatogram of Alfalfa Root Saponins (10 ng).

VITA

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Thesis: ISOLATION AND BIOLOGICAL ACTIVITY OF THE ROOT SAPONINS OF SIX CULTIVARS OF ALFALFA *(MEDICAGO SATIVA* L.) WITH RESPECT TO DORMANCY AND TIME

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