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SAPONIN CONTENT AND SOME POD AND BLOSSOM CHARACTERISTICS OF ALFALFA AS RELATED TO SEED INFESTATION BY THE ALFALFA SEED CHALCID

by

Ronald D. Morse

A thesis submitted in partial fulfillment
of the requirements for the degree

of

MASTER OF SCIENCE

in

Biochemistry and Plant Nutrition

Approved:

UTAH STATE UNIVERSITY
Logan, Utah

1966

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ACKNOWLEDGEMENTS

I am grateful to my major professor, Dr. Keith Allred, for his guidance and encouragement throughout the development of this thesis. I also express my appreciation to the other members of my thesis committee: Dr. Marion Pedersen, Research Agronomist, Dr. David Walker, professor of Horticulture, and Dr. Herman Wiebe, professor of Plant Physiology.

I am grateful to Utah State University, Plant Science Department--project 660-B, for the financial support without which this thesis would not have been possible.

Ronald D. Morse

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INTRODUCTION

The alfalfa seed chalcid, Bruchophagus ruddi Guss., is a jet-black hymenopteran wasp. The destructive nature of this pest has been recognized since the latter part of the nineteenth century. Every year thousands of acres of alfalfa seed are destroyed, with infestation reaching as high as 85 percent in some areas. In Utah the chalcid annually ruins from 5 to 25 percent of the alfalfa seed. Much of this damage goes unnoticed, as infested seed is commonly blown out in the trash during harvesting and cleaning operations. The extent of damage is not restricted to the United States. Wherever alfalfa is grown for seed, the alfalfa seed chalcid is known to have caused considerable reductions in seed yields.

Suitable methods of control have not been developed due to the complexity of this insect with its environment. Chemical control is not feasible because the insecticides which are effective on the chalcid also destroy the insects necessary for pollinating the alfalfa blossoms. Cultural practices are the only methods recommended today and many of these are not practical to the seed producer.

Because chemical control methods have not been found effective, current emphasis is being placed upon finding "resistant" varieties of alfalfa as a solution to the alfalfa seed chalcid problem. Several researchers in Utah and elsewhere have shown that there are significant differences in

chalcid infestation among the alfalfa varieties compared (Minion, 1961; Rowley, 1962; and Strong, 1962).

The specific purpose of this study was to investigate the possible relationships between chemical composition and/or physical characteristics of alfalfa and its susceptibility to the alfalfa seed chalcid. The major objective was broken down as follows: (1) Determine the bulk saponin content in the flowers, pods, leaves, and stems of some selected alfalfa clones. (2) Measure the number of curls per pod, width of the average complete curl, and tightness of the individual curls of the pods of each clone. (3) Determine if the alfalfa seed chalcid shows a preference for the flowers in full-bloom of one alfalfa clone over another. (4) Determine the percent of chalcid infestation of seed from each clone. (5) Calculate the correlation coefficients between these various characteristics of alfalfa and its susceptibility to the alfalfa seed chalcid.

REVIEW OF LITERATURE

Alfalfa Seed Chalcid

Classification of the insect

The present binomial nomenclature of the alfalfa seed chalcid is *Bruchophagus roddi* Gussakovsii. Previous to this classification the name *Bruchophagus gibbus* Boheman was used to identify what was then called the clover seed chalcid. The name clover seed chalcid was commonly associated with all members of the family Eurytomidae whose larval forms feed upon the seeds of alfalfa (*Medicago sativa* L. and *M. falcata* L.), red clover (*Trifolium pratense* L.) and birdsfoot trefoil (*Lotus corniculatus* L.). Rowley (1962) referred to reports from Russia which indicated that in reality there were three different species and that each species of chalcid was restricted to plant species within a particular genus. *B. roddi* Guss., the alfalfa seed chalcid, was reported as capable of infesting only the seeds of alfalfa (*Medicago sativa* L. and *M. falcata* L.), while *B. gibbus* Boh. was reported to be selective to red clover (*Trifolium pratense* L.). *B. kolobovae* Fedoseeva was the species which infested only birdsfoot trefoil (*Lotus corniculatus* L.).

Strong (1962) caged chalcids previously reared on alfalfa and red clover upon plants of both these species and found that chalcids would oviposit only on those plants on which they had been reared. This evidence, along with the work of Neunzig and Gyrisco (1959) on birdsfoot

trefoil, indicated that the name B. gibbus Guss. should no longer be indiscriminately applied to all eurytomids infesting forage legumes. Henceforth, in this thesis, B. roddi Guss. will be the scientific name used to identify the alfalfa seed chalcid.

Growth and development

Emergence. The first adult chalcids that appear in the spring come from seed infested the previous year. Such infested seed may come from nearby volunteer plants or from seed scattered in the field or around the harvesting and storage areas.

Adult chalcid emergence in the Uinta Basin of Utah begins from May 1 to 15, and continues until about July 15 (Sorenson, 1930). The earliest emerged chalcids are males and the population remains predominantly males throughout the season. The first brood begins to emerge from current-season seed crops about July 20 and the second brood emerges approximately one month later. However, emergence is continuous with considerable over-lapping of generations.

Temperature, according to Vinagradov (1941), influences greatly the time of chalcid emergence. His results indicated that the adults emerged when the mean temperature was 64.4 to 68 F, provided the moisture content of the surrounding seed was 15 percent or higher.

Oviposition. After mating, female chalcids seek suitable plants for oviposition. Sorenson (1930) observed that females seek newly formed seeds in a semi-fluid or jelly-like condition and will not oviposit in

seeds after they have reached the dough stage or when the seed materials have started to harden. The positioning of the female is directly over the slight enlargement of the pod caused by the growing seed (Urbahns, 1920).

The requisite semi-fluid stage is when the alfalfa seed is nine days old; there is a decreasing percentage of infestation if the seeds are younger or older than nine days (Strong, 1962). In dissecting thousands of green seeds, Sorenson (1930) found that less than 1 percent contained more than one larva or were infested too late for the insects to complete development before the seeds hardened. In those seeds with more than one larva, either one or both did not complete metamorphosis; hence, only one larva would develop within each seed. When oviposition occurs in seed beyond the optimum stage of maturity for egg laying, the larvae fail to grow, and death often results due to starvation because the seeds become too hard to chew.

In the summer, usually only a few days elapse before the emerging female chalcids are capable of ovipositing. The female is very particular with regard to oviposition and may fly around up to four weeks before locating a suitable alfalfa seed. Females do not migrate extensively when in fields favorable for oviposition. However, Wildermuth (1931) indicates that these insects are strong fliers and may ascend high into the air and be carried by winds to neighboring fields. This might be the case when suitable host plants are not available for oviposition.

To determine the potential offspring of the alfalfa seed chalcid females, Sorenson (1930) dissected 50 gravid females which he had fed in captivity

for 48 hours. The eggs counted from these females varied from 24 to 66 with an average of 42 eggs.

Life cycle. The chalcid overwinters in the alfalfa seed in the larval stage. With the arrival of spring and warmer weather the larvae pupate and transform into adults. In this stage they chew a hole through the seed coat of the alfalfa seed and through the enveloping pod, if it is still surrounding the seed. The adult escapes through these small round holes into the outer surroundings. They usually crawl or fly about the alfalfa plant, mating soon after emergence.

Adult chalcid populations are closely associated with the blooming habits of alfalfa. Adults are rarely found in an alfalfa field which is not in bloom and relatively few are found in the first bloom. Wildermuth (1931) indicates that the adults apparently feed in the alfalfa blossoms and possibly remain alive for several weeks when conditions are favorable.

Upon finding a suitable pod the female adult chalcid oviposits in the seed. These eggs hatch into larvae in 3-5 days and change to pupae in an additional 8-15 days. The pupae emerge as adults in approximately 12 days. Thus, it takes three to four weeks from the egg to the adult stage when conditions are favorable.

Generations per year. Moisture and temperature appear to be the most important factors influencing the number of generations per year. In Utah there are two to three generation per year (Sorenson, 1930). In areas of the western and south western United States where the growing season is much longer, Wildermuth (1931) reported that as many as six generations

per year may occur. Differences in number of generations each year have a decided effect on the extent of damage inflicted by the alfalfa seed chalcid.

Overwintering

The alfalfa seed chalcid overwinters in the larval stage within the alfalfa seed. Neglected fields of alfalfa and volunteer plants which produce seeds along ditches and waste places contribute greatly to the number of overwintering larvae. Chaff stacks, screenings and infested seed pods which have fallen to the ground in alfalfa seed fields also serve as sites for future adult-emergence areas (Urbahns, 1914).

Under favorable climatic conditions the larval stage lasts from 8 to 15 days, but where conditions are too dry at the end of feeding, the larvae may aestivate--go into a resting state. In this condition they may remain within the seeds for periods lasting one or even two years. During a four year period from 1926 to 1929, 24 percent of the larvae of infested seeds produced on first crop alfalfa pupated and emerged as adults the same season. The remaining 76 percent aestivated, hence becoming overwintering larvae. Infested seed from the second crop alfalfa for the same four-year period had approximately 84 percent overwintering larvae. Both summer broods and overwintering broods may emerge during the same period of time. The number overwintering from either crop will change from year to year as environmental factors vary.

Distribution

Damage caused by this insect is not limited to the western United States. On the contrary, the seed chalcid is widely distributed throughout the world wherever alfalfa is grown for seed (Sorenson, 1930). This pest is distributed throughout most of the United States with higher populations found in the irrigated regions of the West and Southwest. Outside the United States the seed chalcid has been reported to cause damage in Germany, Chile, South Africa, and Russia.

Description of the damage

Damage from the alfalfa seed chalcid is not readily apparent in the field. Only by careful observation does one realize that some of the alfalfa seeds may be infested. This "hidden effect" results because the destructive process is carried out entirely within the seed. The tiny larvae feed upon the semifluid or jelly-like albumen of the developing seed (Sorenson, 1930; and Urbahns, 1914). This is approximately the time the cotyledons begin to develop. Feeding progresses quite rapidly after the first two days with most of the inner portion of the seed being eaten, except the seed coat, prior to the normal period of seed hardening.

Infested seeds are usually dwarfed, misshaped and discolored. In very few instances do infested seeds appear normal. When this does occur, they lack the glossy appearance associated with normal seed color. Often the infested seeds appear dusty, as if they were covered with fungal spores, when viewed under the binocular. This dusty appearance is due to a deposition of uric acid excreted by the developing seed

chalcid. Nearly all infested seeds are soft and easily crushed with the fingers. They are also lighter than normal seeds and usually pass out of the harvester in the chaff and screenings. Low seed yield, when a higher yield was indicated by pod set, may be the only indication of chalcid damage unless a careful examination of individual seeds is made before threshing.

Economic importance

Alfalfa seed chalcid has been one of the most serious pests in the production of alfalfa seed for many years. Urbahns (1914) reported the insect increasing rapidly in the United States, causing serious annual losses and in some areas threatening the existence of the alfalfa seed industry. Annual losses in Utah generally range between 5 and 25 percent with an estimated average yearly loss of over 400,000 dollars. Infestations as high as 88 percent have been reported in other areas of western United States (Minion, 1961). In Fresno County, California, losses due to this pest were estimated to be from one to one and one-half million dollars in 1959 (Bacon *et al.*, 1959).

Control

There are three basic methods that could be employed in attempting to control this insect pest.

Natural. Butler and Hansen (1958) list 10 parasites known to affect the alfalfa seed chalcid. All those included are of the order Hymenoptera, superfamily Chalcidoidea. The extent of parasitism varies from season

to season and area to area. Peairs and Davidson (1956) reported that in warmer areas parasites are able to develop nearly as fast as the chalcid; hence, parasitism is increased.

Although parasites can destroy large number of chalcid larvae, their implementation to control the chalcid has not been recommended. The improbability of using natural pests to control the chalcid becomes apparent when one remembers that benefits from parasitism are only important in reducing future chalcid populations, because damage to the seeds has been done before parasitism occurs.

Chemical. Considering the vast present-day knowledge of insecticides, chemical application would appear to be a simple approach in the control of the chalcid. There are several available insecticides that will effectively kill the adult chalcid (Rowley, 1962). However, the problem is how to kill the adult seed chalcid without destroying the necessary pollinators, which are closely related to the seed chalcids and are susceptible to similar types of insecticides. The pollinators further complicate the situation in that they are in the field at the same seasonal time and at the same time of day as the chalcid. Therefore, effective chemical control has not been developed.

Other chemical control methods have and are being tried, such as systemics and soil applications, to kill overwintering larvae. Thus far, nothing of real value has been discovered (Rowley, 1962).

Cultural. At present, practices involving cultural methods are the

only recommended means of controlling the alfalfa seed chalcid. This method, in order to be effective, demands community cooperation. If all recommended cultural practices were applied, the extent of damage could be substantially reduced. Suggested cultural practices are: (1) destroying overwintering larvae by burning or feeding chaff and screenings from harvest and storage sites and by cultivating to bury infested seeds; (2) eliminate all other host plants; (3) grow only one type of host plant in an area; (4) grow either first or second crop for seed (but not both) in one area; (5) manage the seed crop so that blossoming and seed set are as rapid and uniform as possible.

Resistance

Definition and classification

Plants that are inherently less damaged or less infested by insects than others under comparable environmental conditions in the field have been rated as resistant (Painter, 1951).

Painter catergorizes the reported causes of resistance as seen in the field into three bases or mechanisms. Of these, one or a combination of any of the three is present in most cases of resistance that have been studied sufficiently. First, a particular plant may be non-preferred by insects for oviposition, for shelter, for food, or for any combination of the three. Secondly, a given variety may be resistant because adverse effects occur during insect metamorphosis which result when the resistant variety is used for food; this type of resistance is called antibiosis. Thirdly,

resistant plant varieties may show tolerance, or the ability to survive under levels of insect infestation that would kill or severely injure susceptible plant varieties.

Attractants

There appear to be two general ways in which resistance may occur through non-preference: (1) A resistant variety may lack one, or more, or a measurable amount of the qualities which provide the attractive stimuli present in a susceptible variety. (2) A resistant variety may possess repellent qualities which take the place of, or successfully compete with, or mask the attractant stimuli. Of the two, attractants have received considerably more attention during the past few years. Gunther (1960) indicates that the future for attractants looks more promising than does that of repellents for pest control. He states that insects are attracted by attractants a greater distance than they are repelled by repellents. Furthermore, only small amounts of an attractant are usually required to be effective while a larger amount of repellent is necessary to obtain effectiveness.

The theory or mechanism of attractants involves a break in the chain of stimuli which leaves the insect with chance or random trial and error as the means of finding the resistant host. Success in this regard will depend on the size or character of the break and the density of the insect population. Compared to an odor-directed response, the chance method of host-finding would be wasteful of the short lifetime available to the

insect and of the stored food reserves in its body. Some insects die without depositing eggs if a suitable host is not present.

In general, no one attractant alone performs the service of guiding an organism to its proper habitat, mate, or food. The desired end is achieved by a complex array of stimuli working in harmony (Dethier, 1947). The external stimuli governing the time and location of oviposition include temperature, humidity, light, currents, surfaces, odorous substances, and contact with chemical substances. From the point of view of attractants, odors are the most important of these. Not only do they attract graved females, but many, operating alone, actually induce oviposition (Richardson, 1925).

Possible causes of variance in susceptibility

Resistance as a means of control is highly desired from both a convenience and economic point of view. In considering potential host resistance, a major criterion is whether or not the plant species shows consistent variance among varieties in susceptibility to being damaged by the insect pest. Minion (1961) and Rowely (1962) reported that average differences in chalcid infestation were highly significant among several alfalfas compared in Utah.

The literature is skimpy with regard to clues which might explain the occurrence of the variance among varieties in susceptibility to attack by the alfalfa seed chalcid. Painter (1951) generalizes the possible explanations for variance according to the stimuli by which insects find their

plant host. He states that responses to chemical constituents of the plant, to contact with surfaces of the plant, and to colors or intensity of light are the chief means by which insects locate their plant host. As such they are the chief characteristics of the plant which may be modified genetically and hence give rise to resistance by way of a lack of response to plants possessing the modification.

Chemical constituents of alfalfa--saponin. Kamm and Fronk (1964) used an olfactometer to ascertain the odor responses of the chalcid toward many of the known chemical constituents of alfalfa. Depending upon the movement of the insects toward, away, or neither from each constituent when introduced into the olfactometer, Kamm and Fronk classified them respectively as being attractants, repellents, or neutral. Out of 95 chemicals tested, 38 were attractants, 9 were repellents, and 48 were neutral.

Chemicals most attractive were beta carotene, D-ribose, maltose, niacin, vitamin D-2, cholesterol, pangamic acid, alfalfa saponin (medicagenic type), xanthine, diethylstilbestrol, hesperetin, L-histidine, DL-aspartic acid, and L-proline.

Chemicals moderately attractive were L-arabinose, D-galactose, D-xylose, D-pantothenate, folic acid, alfalfa wax, acetyl thiocholine, oxalic acid, dicumarol, beta alanine, and DL-norvaline.

Chemicals slightly attractive were D-pantothenic acid, vitamin B-12, genisten, coumestrol, L-cystine, DL-leucine, DL-glutamine, thiamine, biotin, and DL-tyrosine.

Chemicals slightly repellent were pyridoxine, succinic acid, aconitic acid, coumarin, shikimic acid, xanthophyll, malic acid, and betaine. Butyric acid was highly repellent.

Pedersen (1962) determined the percent bulk saponin in the leaves and stems of 80 varieties and strains of alfalfa. Rowley (1962) reported percent chalcid infestation determinations on the same varieties and strains used by Pedersen. Twelve clones of alfalfa were selected for the author's study in 1964 and 6 of the 12 were taken from varieties that both Pedersen and Rowley had used in their experiments. Table 1 shows the relationship between percent saponin determined by Pedersen and percent chalcid infestation reported by Rowley of these six varieties of alfalfa.

Table 1. Relationship between percent seeds infested by alfalfa-seed chalcid and the percent saponin in the leaves and stems of six varieties of alfalfa

Variety	% Seeds infested	% Saponin in foliage
Rhizoma	54	1.78
Du Puits	51	1.78
Teton	48	1.46
Vernal	44	1.41
Nemastan	38	1.22
Lahontan	37	1.34

In relationship to Kamm and Fronk's (1964) olfactometer studies and the chalcid response to saponin (medicagenic type), significance was achieved when only females were used in the olfactometer. Males alone

showed no significant response and a mixed population of males and females was also non-significant. The experiment was repeated. This time females alone and males alone were significant, but mixed was non-significant. The researchers stated that the mixing of males and females may have masked the attractiveness of saponin by the mere presence of the opposite sex or by their mating habits.

Soya type saponin was also tested in the olfactometer and, although non-significance was recorded in each category, the females when they were not mixed with males, showed a trend toward soya saponin indicating that it was an attractant. This trend approached by 93 percent the statistical value required for significance at the 5 percent level of probability. The statistical analysis used by Kamm and Fronk was a chi square test with 1 degree of freedom. The critical regions were ± 3.84 and the value obtained for soya type saponin was $+3.57$.

Morphological characteristics of alfalfa. Thomas (1963) conducted some experiments in which he attempted to correlate pod morphological characteristics with susceptibility to chalcid damage. He measured the number of curls per pod and the pod thickness of 17 selected varieties of alfalfa. These 17 varieties represented the 10 most susceptible and the 7 most resistant entries as determined by previous studies (Minion, 1961; and Rowley, 1962).

In the number of curls per pod study, those varieties selected for resistance had an average of 2.38 curls per pod. Those selected for susceptibility had an average of 1.92 curls per pod. Statistical computation

showed a close correlation between varieties selected for resistance and the number of curls per pod. A correlation coefficient of -.863 was computed from the 1962 data whereas the 1961 data had a correlation coefficient of -.893 for these two factors. Thomas concluded that this type of resistance may be a "physical resistance." A pod which has many tight curls does not expose as much vulnerable area to chalcid oviposition as an open pod with few curls. Data from this experiment also indicated that the tightness of the curls is a major factor and a pod which has open curls would still be susceptible to infestation even though it may have numerous curls.

The average pod thickness of those varieties selected for resistance to the chalcid was 3.19 units compared to 4.07 units for the more susceptible varieties. Statistical analysis showed a close correlation between varieties selected and pod thickness. A correlation coefficient of .936 was computed from the 1962 data. Data in 1961 had a correlation coefficient of .969 between the same factors.

Rowley (1962) observed and recorded the blossoming habits of 15 alfalfa entries which showed considerable variance in susceptibility to chalcid injury. Some of the alfalfas had a longer blooming period than others; however, all the entries were in full-floom during the period when chalcid numbers were the highest. His results indicated that for most alfalfa entries a longer blooming period apparently had very little influence in relation to percent chalcid infestation. Rowley concluded that something other than the availability of seeds susceptible to chalcid attack is

responsible for the highly significant differences in chalcid infestation.

Color of alfalfa blossoms. One of the most common observations concerning insects is that they respond to light in various ways. Cockroaches scuttle into dark corners when a light is turned on at night, whereas many moths and leafhoppers congregate at various lights. The alfalfa seed chalcid fits into the latter category in that it moves toward light. This particular response can be easily observed by caging chalcids and shading a portion of the container in which the insects were placed. Within minutes most if not all the chalcids will be congregated in the non-shaded portion of the container.

When the behavior of insects with respect to light is analyzed carefully, the different responses of insects to light are associated with the different physical attributes of light--direction, intensity, and color or wavelength.

Painter (1951) states that the responses of insects to plants involve reflected light and the character of the surface from which it is reflected. Therefore, according to this statement, plants with dark colored surfaces would tend to reflect less light than would plants which contain only small amounts of colored pigments. Painter gives numerous examples which illustrate the importance of color as a stimulus for insects in finding their plant host.

Von Frisch (1950) conducted many experiments on the color sense of bees. His results indicated that color, shape, and scent of the flowers were all very important guides to bees in finding the desired flower for

acquiring food. The color of flowers has the advantage that it can attract bees from a greater distance, while scent is specific for each species and thus permits the definite recognition of the flowers at close range. Further results showed that white light, containing all wave lengths visible to bees, is not striking for them; the light must apparently be colored in order to be attractive. In this connection the human eye can distinguish about 60 distinct colors in the visible spectrum, while the bee apparently sees only four different colors: yellow, blue-green, blue, and ultraviolet.

Saponin

Occurrence in plants

Plants containing saponins are widely distributed in the plant kingdom. At least 400 plant species belonging to 50 different families produce saponins. Saponin is probably formed in the leaves but is found in all parts of the plant and occurs very often in high concentration in the seeds (Taylor, 1965).

Uses

Saponins were originally named because of their soap-like properties. They have been used as detergents and in foam-type fire extinguishers. They have also been employed as wetting agents in the textile industry (Lindahl *et al*., 1957).

In very dilute solutions they are quite toxic to fish, and plants containing them have been used as fish poisons for hundreds of years.

Certain saponins have become important in recent years because they may be obtained in good yields from some plants and are used as starting material for the synthesis of steroid hormones (Robinson, 1963) and oral contraceptives (Djerassi, 1966).

Chemistry

Two types of saponins are recognized--glycosides of triterpenoid alcohols and glycosides of a particular steroid structure described as having a spiroketal side chain. Both types are soluble in water, pyridine, and ethanol, but are insoluble in acetone, benzene, and ether. Their aglycones, called sapogenins, are prepared by acid or enzymatic hydrolysis and without the sugar residues have solubility characteristics of other sterols.

Saponins are powerful surface active agents which cause foaming when shaken in water. When added to blood in low concentrations saponins may produce hemolysis of red blood cells. This high surface activity is a property of saponin because the sapogenin is fat-soluble while the sugar moiety of the intact molecule is water soluble (Lindahl *et al.*, 1957).

Mangan (1959) studied the effect of pH on the foaming properties of saponin. His results showed that foam strength responded markedly to changes in pH. Above pH 6.0 foam strength was nil, but rose below pH 5.5 to a sharp peak with an optimum in the region of 4.5 to 5.0. On further acidification foam strength dropped rapidly to almost nil at pH 4.0.

Foam expansion shows a similar optimum in the same pH region but not to the marked extent as foam strength. Thus above pH 6.0 alfalfa saponin gives an appreciable volume of foam which, however, exhibits little resistance to mechanical stress. Mangan also showed that the presence of calcium was necessary for the formation of strong saponin foams.

Alfalfa saponins are considered to belong to the triterpenoid group. Crude saponin isolated from alfalfa is complex in nature and its components are very difficult to separate. Hanson et al., (1963) mentioned that upon hydrolysis, mixed alfalfa saponin will yield a mixture of triterpenoid sapogenins, sugars, uronic and galacturonic acids. Glycosylation is generally at the carbon-3 position and several different monosaccharides are usually present as an oligosaccharide. Several different saponins may all have the same sapogenin but different sugars (Robinson, 1963).

Various researchers have reported on the sapogenin constituents in alfalfa. Separate studies by Potter and Kummerow (1954) and Bevenue and Williams (1955) showed that there were three genins in alfalfa. Lindahl et al., (1957) reported seven different sapogenin fractions recovered from alfalfa. Although physical constants were determined for each fraction, chemical identification was made on only three--soyasapogenols A, B, and C. Isolation of a new triterpenoid was accomplished in 1959 (Livingston, 1959). It was a trihydroxy, monolactone, monocarboxylic acid and was named lucernic acid. Djerassi et al., (1957) and Morris et al., (1961) isolated and identified another sapogenin in alfalfa which

they named medicagenic acid.

Willner et al., (1964) have spent a considerable amount of effort in the attempt to separate and identify the sapogenin constituents of soybeans. Their results indicated that there are five different soyasapogenols in soybeans. These five were subsequently named soyasapogenols A, B, C, D, and E.

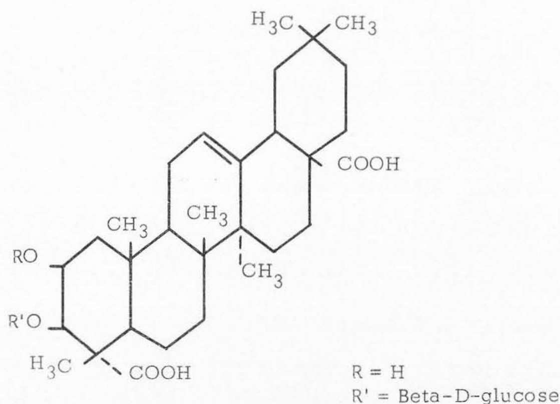
The literature also varies considerably regarding the number of saponins in alfalfa. A summary of research done up to 1957 would indicate that at least six saponins, and possibly several more, occur in alfalfa (Lindahl et al., 1957). Seven constituents of alfalfa saponin were found by paper chromatography by Coulson (1958). However, similar experiments by Lourens and O'Donovan (1961) revealed eight saponins. More recent chromatographic studies have shown that there are 10 possibly 12 constituents of alfalfa bulk saponin (Coulson and Davies, 1962).

There appear to be two explanations for the deviations in the number of reported saponins in alfalfa. First, there is an inherent quality difference among alfalfa varieties which would cause one variety to have a different number of saponins than another. Secondly, because one sapogenin may combine with more than one type of sugar, many different saponins could occur within a particular variety. The limiting factors in this case would be the diversity of the sapogenin and sugar constituents and the presence of the requisite chemical environment for their combination.

Sapogenin biosynthesis

While not all of the fine details relative to the biosynthesis of the sapogenins have been worked out, the main pathway appears to be established, and involves a succession of reaction: Acetyl Co A → Acetoacetyl Co A → Beta - hydroxy - beta - methylglutaryl Co A → Mevalonic acid → 5-Phosphomevalonic acid → 5-Diphosphomevalonic acid → Isopentyl pyrophosphate ⇔ 3, 3-Dimethylallyl pyrophosphate → Geranyl pyrophosphate → Farnesyl pyrophosphate → Squalene → Sapogenins. Each reaction requires specific cofactors and enzymes which are not included in this biosythesis scheme. Also a number of stages may be involved in several of the foregoing reactions. In the final step the triterpenoid sapogenins are derived from all trans-squalene by a series of concerted cyclization and rearrangement reactions (Richards, 1964).

The parent triterpenoids contain 30 carbon atoms and have carbon skeletons which are divisable into six isoprene units. The exact chemical structure of all the reported different triterpenoid sapogenins found in alfalfa have not been determined. Willner et al., (1964) have done some work toward the elucidation of the soyasapogenolic structure. Morris, Dye, and Bisler (1961) structurally identified an alfalfa root saponin named medicagenic acid. Chemical constants, elemental analysis, hydroxyl determinations, infrared interpretations and NMR study provided evidence for the following structure:



R = H
R' = Beta-D-glucose

Physiological and pharmacological effects

The effect of saponin in dilute solutions on water living animals has been mentioned previously. In particular tadpoles and fish are strongly poisoned. Turova and Gladkikh (1963) have written a review of literature of the experimental and clinical pharmacology of saponins. Some of the reported effects of saponin according to Turova and Gladkikh are: (1) irritants; (2) enhancement of the absorption of foodstuffs and drugs from the intestines; (3) antisclerotic agents; (4) pronounced actions on the cardiovascular, nervous, and digestive system.

Principle symptoms of intoxication by saponins are nausea, vomiting, vertigo, ataxia, not frequently diarrhea, and loss of appetite (Turova and Gladkikh, 1963). When administered through the mouth the saponins are a hundred times less toxic than by an intravenous administration.

Kingsbury (1964) includes alfalfa as a poisonous plant because it contains saponin. The response of many animals to saponin from alfalfa bears this out. Saponin as a causal agent of bloat in ruminants has been considered for some time. Maclay and Thompson (1956) pointed out that alfalfa from farms where bloat occurred contained more saponin than alfalfas which did not produce bloat. Lindahl et al.,(1957) found that 50 grams of alfalfa saponin administered intraruminally caused death while one gram given intravenously had the same fatal effect in mature range sheep. The saponins were found to interfere with normal ruminal motility and eructation. Severe damage to various internal organs was revealed by autopsies performed on the sheep that died. Different animals varied considerably in the reaction to the alfalfa saponins, some being very sensitive while others appeared to be quite resistant. The authors concluded that saponin was only one of several factors which might cause bloat.

A respiratory inhibitor was isolated from alfalfa meal by Shaw and Jackson (1959). The behavior of the inhibitor during isolation suggested it to be a saponin. Coulson and Davies (1962) used strips of rat diapharm muscle to measure changes in respiration when various levels of alfalfa saponin were used. The reduced oxygen uptake by the muscle tissue in the presence of the saponin indicated that saponin is a respiratory inhibitor.

When saponin was included in the diet of baby chicks at less than 0.5 percent, a depression in growth occurred (Heywang and Bird, 1954).

Peterson (1950a, 1950b) and Potter and Kummerow (1954) conducted experiments with baby chicks in a manner similar to Heywang and Bird. Both sets of researchers reported that the saponin used caused a reduction in the growth of baby chicks.

Laying hens were adversely affected by a 0.4 percent supplement of alfalfa saponin as well as by a 20 percent supplement of alfalfa meal to their ration (Heywang, Thompson, and Kemmerer, 1959). The results showed a reduction in the number of eggs laid. However, another experiment (Anderson, 1957) indicated that the reduction in egg production was only temporary.

McGuire (1965) cites many experiments in which it was demonstrated that saponin is a germination inhibitor of many species of plants such as cotton, corn, wheat, and sorghum. Lawrence and Kelcher (1962) tested 14 different root extracts each on 15 different plant species. Their results indicated that alfalfa root extract was the most toxic to seed germination and seedling growth on the 15 plant species.

Methods for determining bulk saponin content

Methods that have been used for recovering saponins vary almost as widely as the plants from which they are obtained. Many reagents, such as various metal salts and tannins, are reported to precipitate these compounds from aqueous or alcoholic solutions. However, this method of recovery may be far from complete and often results in alteration of the saponins (Lindahl *et al.*, 1957).

The characteristic properties of saponins have been useful in devising analytical methods for saponin determination. Saponins are known to produce a frothy foam when shaken in water. Glover and Stanford (1963) and Kendall (1964) devised methods for measuring foaming properties of forages. This method lacks accuracy because several other factors besides the saponin content influence foam strength or foam volume. The hemolytic nature of saponins suggested another method of analysis (O'Dell et al., 1959; and Birk et al., 1963). A spectrophotometer was used to measure the varying amount of hemolysis caused by different saponin concentrations.

The tendency of cholesterol to form addition compounds with saponins has been used to recover saponins from alfalfa (Lindahl et al., 1957), but attempts to base an analytical method on this principle were abandoned when preferential recovery of some and incomplete recovery of all alfalfa saponins could not be avoided. For a time a method was used whereby sapogenins rather than intact saponins were estimated. However, the procedure required much precise manipulation and far too much time for routine application.

The Liebermann-Burchard reagent is known to produce characteristic colors when mixed with saponin (Van Atta and Guggolz, 1958). However, this reagent which consists of an equal mixture of concentrated sulfuric acid and acetic acid gives an unstable and irreproducible color. Gestetner et al., (1963) using a soybean saponin extract devised a colorimetric method in which they replaced acetic anhydride with glacial acetic acid.

The color reaction was stable for at least 30 minutes which enabled them to determine the quantitative saponin content of the soybean extract. A colorimetric procedure has been used by Van Duuren (1963) which also employed a modification of the Liebermann-Burchard reagent. He hydrolyzed saponin from sugar beet products, then treated the resulting sapogenin with a mixture of acetic acid, acetic anhydride and sulfuric acid to produce the colored complex. The intensity of the color was measured and related to the percentage of saponin present. A relatively pure saponin could be measured by this technique.

The separation of the saponin components is complicated by their similarity of properties. However, this is advantageous because it allowed a technique to be developed which measures the entire complex of saponins in alfalfa (Van Atta, Guggolz, and Thompson, 1961). This procedure has been referred to as the "carbon-pyridine" method for determining the bulk saponin content in alfalfa. With this method, complete recovery of saponins was demonstrated; however, results were known to be higher than the true values. The desirability of a more accurate total saponin method influenced Van Atta (1962) to develop a supplement to the carbon-pyridine procedure. By this supplementary treatment, inaccuracies due to incomplete separation of saponins and non-saponins can be considerably reduced.

McGuire (1965) utilized the seed inhibitor nature of saponin as a method of analysis. McGuire developed a bio-assay technique in which

he compared the saponin content with the degree of inhibition of lettuce seed. Using the carbon-pyridine procedure as a standard comparison for saponin content, the results indicated that saponin content of alfalfa can be estimated by measuring the degree of lettuce seed inhibition when water extracts are the source of saponin. This conclusion is more valid within a single variety than when data from many varieties are compared. There appeared to be quality as well as quantity differences in saponin that affect seed inhibition and these quality differences seem to vary among varieties.

Many surface-active chemical compounds are known to possess fungi-static properties. Zimmer *et al*., (1966) recognized that saponin is a surfacant and subsequently developed a bioassay technique for bulk saponin estimation. Through experimentation, *Trichoderma viride* was found to be particularly sensitive to saponin. A correlation value of -.9525 between the saponin content as determined by the carbon-pyridine procedure (Van Atta *et al*., 1961) and the growth of *T. viride* on media containing 5 percent alfalfa leaf meal extract clearly demonstrates the value of this test for estimating the saponin content of alfalfa. This procedure is relatively fast and would especially be applicable when many samples were to be tested or when rapid or preliminary estimation was desired.

Factors affecting saponin content in plants

A detailed study by Hanson *et al*., (1963) indicated that the saponin

content of alfalfa is influenced by both genetic and environmental factors. Their results showed the effects of locations, varieties, and cuttings to be all highly significant. These researchers concluded that with the variation observed among varieties of alfalfa, it would seem reasonable to be able to change the saponin content by breeding. The extent to which this could be done would depend on the genetic variation among individual plants.

This same study showed that saponin was significantly and positively correlated with protein, ash, fat, and nitrogen-free extract and significantly but negatively correlated with crude fiber and hay yield.

METHODS AND MATERIALS

Establishing Field Plots

A world collection of alfalfa clones has been grown at the Evans Farm near Logan, Utah, for experimental purposes over the past several years. In previous studies these clones were evaluated for relative resistance to attack by the alfalfa seed chalcid. During the summer of 1964, vegetative cuttings were taken from 11 of the alfalfa clones. These 11 clones represented the entire span of susceptibility ranging from the highest to the lowest in percent alfalfa seed chalcid infestation. One clone was also selected from an alfalfa nursery on the Greenville Farm, north of Logan, because it was considered to show low susceptibility to alfalfa weevil (Table 2).

Several cuttings of each plant clone were maintained in pots containing moist sand until they had developed a root system. They were transplanted into pots containing soil and were grown in the greenhouse until June, 1965, at which time some of each clone were taken to the Evans Farm for transplanting. The soil at the Evans Farm was a Nibley silt loam. The field design was randomized block with eight replications and four plants of each clone per replication. The plants were spaced on two foot centers. The design contained eight replications arranged linearly from east to west with two border rows of alfalfa surrounding each one.

Table 2. Origin of 12 clones of alfalfa used in alfalfa seed chalcid resistance studies

Clone no.	Clonal origin (description)
1	Rhizoma Registered Can. Cert. 2299
2	Du Puits FC 24340
3	Iraq P.I. 217,648
4	Teton S.D. (1959) FC 35346
5	Vernal Cert. W-52 N. K.
6	Utah 39 Syn C-2 (1959)
7	Nemastan (1946)
8	Lahonton Cert. FF 0643 N.K. (1959)
9	Afghanistan P. I. 212,104
10	Iran (A)[a] P.I. 222,178 (Utah:201-c)
11	Iran (B)[a] P.I. 222,178 (Utah:201-d)
12	Iran (C)[a] 228,350---1282

[a]The letters A, B, and C were added to clones 10, 11, and 12 for convenience purposes only.

Sufficient plants of each clone were left in the greenhouse so as to maintain a supply in case injury or loss occurred to the field plants.

The initial forage growth was cut off and removed July 8, 1965. The plants were then allowed to grow, mature, and produce seed. The alfalfas were sprayed with dieldrin on July 20, and with dylox on July 14 and July 24, to control weevil and lygus bugs respectively. The plots were irrigated at regular intervals to maintain the plants in an actively growing condition.

Harvesting and Preparing Plant Samples

Racemes

Racemes containing flowers in full bloom were collected from the third

and fourth plants of each clone in six replications and dried at approximately 135 degrees F in a forced-air oven for 24 hours. The racemes were picked on four different occasions between August 11 and September 1, 1965. After drying, the blossoms were stripped from the racemes, ground in a Wiley mill, and stored in plastic vials.

Dark-green pods

Dark-green pods at the 7-11 day old stage of development were harvested from the first and second plants of each clone in the first six replications. Harvesting was done on four different dates from August 6 through September 9, 1965. The pods were dried in like manner as were the racemes. Flower fragments were removed from the pods and discarded, then the pods were ground in a Wiley mill and stored in plastic vials.

Leaves and stems

The entire fourth plant of each clone in the first four replications was taken during a six day period from August 27 to September 1, 1965. Drying was accomplished in the same manner as above with the racemes and dark-green pods. Following drying, all pods, buds, and flowers were removed from the foliage and discarded. The foliage was separated into leaves and stems, ground individually in a Wiley mill and stored in vials.

Mature pods

On September 24, 1965, the second, third, and fourth plants of each

clone in the first few replications were harvested and dried. The fully mature pods were stripped off and saved for a determination of infestation by the alfalfa seed chalcid.

Bulk Saponin

The carbon-pyridine procedure as developed at the Albany laboratory (Van Atta *et al.*, 1961; and Van Atta, 1962) was used in determining the total saponin complex in the alfalfa samples. This method has been employed by other researchers at Utah State and elsewhere (Pedersen, 1962; and Hanson *et al.*, 1963).

Sample preparation

A 6-8 gram sample of plant material from the plastic vials was dried in an open container in a vacuum oven at 65 degrees C for 16 hours and then placed in a desiccator. A 4 gram portion of the vacuum dried sample was weighed, transferred to a 250 ml Erlenmeyer flask where it was mixed with 20 ml of water and allowed to stand for five hours. Fifty-five ml of 95 percent ethyl alcohol was added, mixed by swirling, and let stand for 16 hours (overnight). Next, 15 ml of 95 percent ethanol and 43 ml of water were added, mixed, and allowed to equilibrate for one hour. This mixture was then suction filtered using Whatman Number 1 filter paper.

Recovery of saponins

One gram of activated carbon (cf. carbon selection and reactivation) was added to a 50 ml portion of the ethanol extract and warmed gently

over steam with occasional stirring for 15 minutes. The solution was suction filtered through Whatman Number 1 filter paper precoated with celite filter aid. After filtering, the carbon-containing filter paper was washed with 100 ml of 50 percent ethanol and the filtrate and washings were evaporated over a steam bath to near dryness to drive off the alcohol. Prolonged or overheating at this point will lead to discolored end products and high analytical yields.

To the evaporation residue, which is like a thick syrup in appearance, was added 20 ml of distilled water. After the residue was dissolved completely in the water, 1.5 grams of activated carbon was added and the mixture was stirred occasionally while being warmed gently over the water bath for five minutes. The solution was suction filtered through Whatman Number 50 filter paper precoated with approximately 0.5 grams of filter aid. The "filter-cake" was washed three times in succession with 70 ml distilled water, 20 ml of 10 percent ethanol, and 20 ml of 20 percent ethanol, respectively. Both the filtrate and the washings were discarded.

To elute the absorbed saponins from the carbon, the filter cake was washed with 200 ml of a mixture of pyridine (purified grade) and absolute ethanol--3 to 7 (V/V). The pyridine-alcohol eluate was evaporated over steam in a tared dish. Following complete evaporation, the dish containing the saponin residue was vacuum dried for 16 hours at 65 degrees C, cooled in a desiccator, and weighed.

Throughout the final carbon addition step, which involves the

filtration, washing, and elution processes, the level of the liquid was not permitted to reach the surface of the filter cake between additions. This precaution was necessary in order to prevent the recording of low saponin yields.

To determine milligrams of saponin per gram dry-weight of alfalfa sample, the milligrams of saponin residue obtained from the carbon-pyridine procedure was divided by a factor 1.5. The divisor represents the grams of dry matter in a 50 ml portion of the ethanol extract.

Determination of percent saponins

The percentage of bulk saponin recovered from alfalfa samples is higher than the true values. Van Atta (1962) developed a supplementary treatment in which inaccuracies due to incomplete separation of saponins and nonsaponins can be considerably reduced.

For brevity, the impure saponins eluted from carbon with ethanol and pyridine in the preceeding procedure are referred to here as CAP saponins. The term CAP stands for the carbon-pyridine method of saponin determination as it has been described above.

Chromatogram application. CAP saponins while still in the dish in which they had been weighed, were dissolved in 1 to 2 ml of 60 percent ethanol. With a streaking pipette, this solution was applied to a sheet of Whatman 3 MM paper in a streak parallel to and 7 cm from the bottom of the paper. Best results occurred when the streak was about 30 cm long and the density was about 1 mg of CAP saponins per cm of streak.

When streaking was completed, the pipette was rinsed with 60 per cent ethanol. Rinsings were delivered into the dish that initially contained the CAP saponins, the liquid was evaporated, and the dish and residue were vacuum dried at 65 degrees C and weighed to determine by difference the weight of CAP saponins applied to the paper.

Chromatogram development. After the length of the streak had been measured and recorded, the width of the paper was accurately trimmed to the ends of the streak, and a descending chromatogram was developed on the paper. The developing solution used was the upper phase of a n-butanol--1 M ammonium hydroxide--95 percent ethanol (60:30.5:13) solvent mixture. Development was stopped when the liquid front had advanced 15 cm below the level of sample streak.

Recovery of saponins. Saponins were located on the dried chromatogram by staining a 1-cm wide test strip that was cut from the top to the bottom of the chromatogram at its middle. The strip was stained by drawing it through a mixture of sulfuric acid and acetic anhydride--1 to 1 (V/V). Positions of saponins became visible on the test strip by the appearance of colored bands in the region between about R_f O.55. The stained portion was then used as a guide to locate the position of the saponins on the remaining unstained portion of the chromatogram. When illuminated by a long-wave ultra-violet lamp, these saponin regions displayed a number of horizontal fluorescent bands which Van Atta (1962) concluded were probably produced by phenolic substances. Using appropriate fluorescent bands as guides, the marginal marks at the upper

and lower limits of the saponin zone were extended across the entire chromatogram.

The area containing the saponin zone was removed and cut into small pieces. Saponins were recovered from the pieces by warming them gently for five to seven minutes with each of three successive 40 ml portions of 60 percent ethanol. The extract solutions, recovered by draining, were combined, filtered, and evaporated. The evaporation residue was vacuum dried at 65 degrees C and weighed. A correction for the soluble substances extracted from the paper itself was deducted from the weight of the residue. The size of the correction was determined from the weight of residue obtained when a blank paper was developed, sectioned, and extracted in the same way as the chromatogram.

The weight of CAP saponins applied to the part of the chromatogram taken for saponin recovery was calculated from the total weight of sample applied, the total length of the sample streak and the width of the test strip. The purity of the CAP saponins--i.e., the approximate percent of saponins in the sample--was calculated from the weight of material recovered from the chromatogram, corrected for the paper blank, and from the sample weight it represented.

Comments on supplementary method. Estimates of saponin percentages obtained in the manner described here are not completely free from inaccuracy. Evidence for this is the slight color of the final products caused presumably by fluorescent non-saponins co-existent with saponins in the same zone on the chromatogram. The author of this procedure

(Van Atta, 1962) attempted to determine the magnitude of the error introduced by the presence of fluorescent materials in the saponin fraction recovered from the paper. He was unable to determine the amount of error with certainty, but concluded that all results done at the Albany laboratory suggested that it cannot be significant.

Carbon selection and reactivation

Preliminary trials conducted by Taylor (1965) indicated that the best carbon source was Nuchar C-115-N, obtained from Industrial Chemical Sales, 230 Park Avenue, New York 17, New York.

The carbon was reactivated according to the method as recommended by Van Atta et al.,(1961). One-hundred and fifty grams of carbon were added to 3 liter of 1 N HCl and the mixture was heated to 80 C. The carbon-acid solution was held at 80 C for 30 minutes, suction filtered, and washed with 9 liters of distilled water. The filter cake was drained, transferred to a larger container, mixed with 15 liters of distilled water, and stirred to dissolve the carbon lumps. This mixture was filtered as before and washed repeatedly with distilled water until the washings were free of HCl as shown by tests with indicator paper. Care was taken during both washing steps to avoid complete drainage of the cake until all the water was added.

The cake was drained, transferred to an enameled pan, and reactivated by being heated for 15 hours in an oven set at 105 C. The carbon was transferred to pint bottles which were kept tightly closed when

carbon was being withdrawn for use.

Alfalfa Morphological Characteristics

Between August 20 to September 1, 1965, one plant from each clone in the first four replications was harvested one at a time. Five racemes containing pods at the 7-11 day stage of development and 10 racemes containing blossoms in full bloom were selected at random from each harvested plant.

Using a method of random numbers, one pod was selected from each raceme and the number of curls, the width of the average complete curl, and the tightness of the curls were measured with a ruler graduated into millimeters. To facilitate the measurement of the tightness of curls a calibrated binocular was used which was graduated into units equaling 0.8 mm.

Alfalfa Blossom Color

Blossom color rating

Blossoms of the 12 alfalfa clones used in this study showed a wide range in coloration. A system was devised using a range scale from 1 to 10 for rating each clone according to its relative concentration of purple or blue blossom pigments. A value of 1 was used for blossoms containing a relatively high concentration of purple or blue pigments whereas a value of 10 represented blossoms containing no visible dark pigments. Six different individuals, each working separately and apart from the other

five, rated the blossoms of each clone. The rating was done at the Evans Farm on August 5, 1966. On this date, the flowers of all 12 clones were in full-bloom.

Blossom preference

In order to determine whether or not the alfalfa seed chalcid preferred blossoms of one alfalfa clone over another, a container was built into which blossoms of each clone and numerous chalcids were placed. The container was a handmade, clear plastic Petri-type dish 40 cm in diameter and 4 cm high.

The procedure began by obtaining fresh alfalfa blossoms from plants in the greenhouse each morning. Four racemes from a clone were arranged erect on the lid of a small plastic vial with the peduncle of each raceme extending into the vial through a small hole in the lid. The vials were filled with water and placed randomly inside the Petri dish at its periphery (Figure 1). One hundred to 150 adult chalcids were captured each morning from the fields; these were placed inside the container. After a one hour adjustment period, the number of chalcids present on the blossons for each clone was counted at the commencement of eight 20 minute intervals. The total of the eight countings was recorded as the number of chalcids visiting each clone for that replication.

The experiment was repeated on five different occasions from July 28 to August 2, 1966. The percent of chalcids visiting each clone during a replication was calculated by dividing the total number of chalcids which

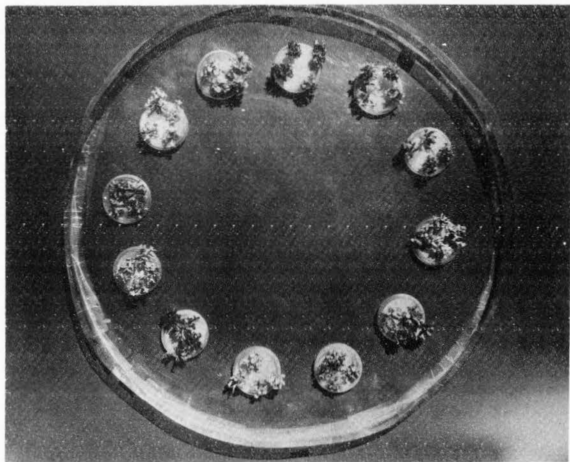


Figure 1. Plastic Petri-type dish, containing the flowers from 12 alfalfa clones, used in blossom-preference study

visited the alfalfa blossoms of all 12 clones combined, into the number visiting each clone. Some chalcids were not visiting the blossoms at the time counts were made.

Care was taken to select equal quantities of flowers from each clone and to use only those flowers which were in full-bloom and yet not wilted. Also in an attempt to avoid bias selection, flowers were taken from several different plants of the same clone which were spaced throughout the greenhouse in a randomized block design.

The same procedure as outlined above was also used to compare clones two at a time. Four clones representing the opposites in flower color were selected for this study. Clones 1 and 5 were used because they had light colored blossoms whereas clones 2 and 9 contained relatively high concentration of purple pigments in their blossoms. All possible two-way combinations of these four clones were tested to determine blossom preference. This amounted to six separate comparisons which were replicated three times. Four vials of each clone were used per replication, hence making a total of eight vials of blossoms which were placed in the Petri dish for each individual experiment. The blossoms used in these six experiments were taken from the plants located in the field plots at the Evans Farm.

Determination of Percent Alfalfa Seed Chalcid Infestation

Mature pods of each clone were harvested on September 24, 1965 from the experimental plots at the Evans Farm (cf. harvesting samples

section). The method of random selection used was to place the mature pods in a narrow V-shaped container and after the pods were mixed thoroughly they were pushed toward the V-end of the container. As the pods appeared at the small opening, they were shelled one by one and all the seeds of each pod selected were analyzed for chalcid damage with the aid of a binocular. Sufficient pods were analyzed until 100 seeds had been obtained. This process was repeated for each clone in each of the first four replications.

Statistical Analysis

The data for each variable in this study were analyzed using the standard analysis of variance (in a completely randomized design) and Duncan's New Multiple Range Test. Simple linear regression analysis was used to obtain the correlation coefficients for all possible two-way comparisons between variables.

RESULTS AND DISCUSSION

Alfalfa Saponin

Percent bulk saponin

Saponin content of the flowers, pods, leaves, and stems of the 12 clones of alfalfa are presented in Table 3. Additional data concerning the calculation of percent saponin are presented in the appendix. A highly significant difference in percent saponin was found among the 12 clones for each of the four plant parts.

Clones 1, 2, and 6 ranked consistently high in saponin for all plant parts. Clone 4 contained a large amount of saponin in all parts except in the pods where it ranked tenth. Clones 9, 10, and 12 most frequently had low concentrations of saponin except clone 9 which was ranked fifth highest in saponin percentage of the leaves. These differences in saponin percentages can best be seen by referring to Table 4.

The saponin concentrations in the stems of the 12 alfalfa clones were considerably lower than in the other plant parts analyzed. The average percent saponin in the flowers, pods, and leaves was respectively 6.5, 3.6, and 3.8 times more than that in the stems.

In order to derive an estimate of the saponin potential for each clone, the saponin percentages of the flowers, pods, leaves, and stems were totaled and the sum divided by four (Table 5). There was a highly significant difference in the average saponin content calculated for the 12 clones.

Table 3. Ranked means of percent saponin in the flowers, pods, leaves, and stems of 12 clones of alfalfa

Plant parts	Clone number	Rank	Clonal origin	Average % saponin	Lest significant ranges 5% level Duncan's new multiple range test
Flowers	2	1	Du Puits	3.58	A
	4	2	Teton	3.55	A
	11	3	Iran B	2.76	B
	1	4	Rhizoma	2.71	B
	6	5	Utah 39	2.64	B
	8	6	Lahontan	2.43	C
	3	7	Iraq	2.42	C
	10	8	Iran A	2.31	C D
	7	9	Nemastan	2.28	C D
	5	10	Vernal	2.23	D
	9	11	Afghanistan	2.19	D
	12	12	Iran C	2.17	D
			$\bar{X}$	2.61	
			F value for clones		100.67**
			Coefficient of variation		.035
Pods	1	1	Rhizoma	1.97	A
	2	2	Du Puits	1.82	B
	11	3	Iran B	1.69	C
	7	4	Nemastan	1.51	D
	8	5	Lahontan	1.48	D
	6	6	Utah 39	1.44	D E
	5	7	Vernal	1.42	D E
	12	8	Iran C	1.34	E F
	3	9	Iraq	1.28	F G
	4	10	Teton	1.23	F G H
	10	11	Iran A	1.16	G H
	9	12	Afghanistan	1.13	H
			$\bar{X}$	1.46	
			F value for clones		44.86**
			Coefficient of variation		.048

Table 3. Continued

Plant parts	Clone number	Rank	Clonal origin	Average % saponin	Lest significant ranges 5% level Duncan's new multiple range test
Leaves	4	1	Teton	2.21	A
	1	2	Rhizoma	1.97	B
	6	3	Utah 39	1.96	B
	2	4	Du Puits	1.64	C
	9	5	Afghanistan	1.49	D
	7	6	Nemastan	1.48	D
	5	7	Vernal	1.44	D
	3	8	Iraq	1.42	D
	8	9	Lahontan	1.35	D E
	11	10	Iran B	1.20	E F
	10	11	Iran A	1.16	F
	12	12	Iran C	1.12	F
			$\bar{X}$	1.53	
			F value for clones		63.41**
			Coefficient of variation		.051
Stems	4	1	Teton	.55	A
	2	2	Du Puits	.45	B
	6	3	Utah 39	.45	B
	1	4	Rhizoma	.42	B C
	12	5	Iran C	.42	B C
	9	6	Afghanistan	.41	B C
	3	7	Iraq	.40	B C
	5	8	Vernal	.37	C
	8	9	Lahontan	.37	C
	11	10	Iran B	.37	C
	10	11	Iran A	.36	C
	7	12	Nemastan	.25	D
			$\bar{X}$	.40	
			F value for clones		13.31**
			Coefficient of variation		.084

**Significant at the 1 percent level of probability.
[a]Significant difference exists between any two means not found in the same range.

Table 4. Summary of saponin data that has been transformed to show the percent of difference among the 12 clones of alfalfa for each of the four plant portions[a]

Clone number	Clonal origin	Percent of the highest saponin value flowers	pods	leaves	stems	average
1	Rhizoma	76	100	89	76	85
2	Du Puits	100	92	74	81	87
3	Iraq	68	65	64	73	68
4	Teton	99	62	100	100	90
5	Vernal	62	72	65	67	67
6	Utah 39	74	73	89	81	79
7	Nemastan	64	77	67	45	63
8	Lahontan	68	75	61	67	68
9	Afghanistan	61	57	67	75	65
10	Iran A	65	59	52	65	60
11	Iran B	77	86	54	67	71
12	Iran C	61	68	51	76	64

[a]The transformation value for each clone was computed mathematically by giving a value of 100 to the clone with the highest percent saponin and comparing the ratio of the percent saponin of each clone to that of the highest. This same method was used for each plant part.

Table 5. Ranked means of the average of the percent saponin in the flowers, pods, leaves, and stems of 12 clones of alfalfa

Clone number	Rank	Clonal origin	Average % saponin in plant part	Least significant ranges[a] 5% level Duncan's new multiple range test
2	1	Du Puits	1.90	A
4	2	Teton	1.88	A
1	3	Rhizoma	1.78	B
6	4	Utah 39	1.62	C
11	5	Iran B	1.51	D
8	6	Lahontan	1.41	E
3	7	Iraq	1.40	F
5	8	Vernal	1.39	F
7	9	Nemastan	1.39	F
9	10	Afghanistan	1.30	G
12	11	Iran C	1.26	G H
10	12	Iran A	1.24	H
		$\bar{X}$	1.50	
		F value for clones		230.39**
		Coefficient of variation		.018

**Significant at the 1 percent level of probability.

[a]Significant difference exists between any two means not found in the same range.

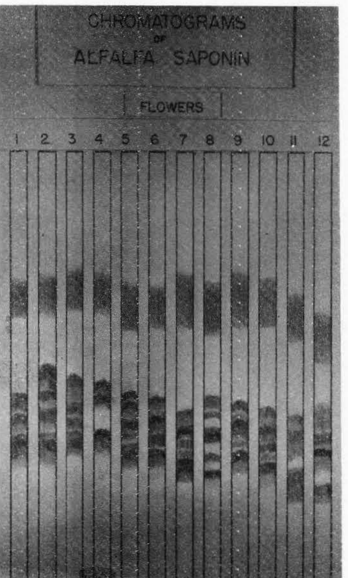
Quality differences in saponin

Descending paper chromatograms of alfalfa saponin were run for each clone in all four portions of the plant. The application procedures, solvent system, development, and detecting of the chemicals on the paper were the same as described in the supplementary procedure for saponin determination. However, in order to further separate the saponin constituents, the chromatograms were allowed to develop for 30 hours.

The chromatograms showed apparent saponin quality differences as evidenced by the varing numbers, colors, and arrangements of the saponin bands (Figures 2 and 3). The number of bands ranged from 8 to 12 for flowers, 5 to 9 for pods, 3 to 5, and 4 to 5 for leaves and stems respectively. The saponin bands stained many different colors ranging from purple through pink in the color spectrum. The arrangement and color of the saponin bands among the 12 clones also varied for each plant part. For example, clone 9 did not have any blue colored saponin bands in the pods whereas the other 11 clones did. Also, the stems of clones 5 through 12 and the leaves of clones 8 through 12 had no saponin bands that stained blue. Yellow bands appeared for all clones in the stems and for clones 7 through 12 in the pods while no such bands appeared elsewhere.

Morphological Characteristics of Alfalfa

Data for three morphological characteristics of alfalfa are presented in Tables 6, 7, and 8. They include curls per pod, width of the average



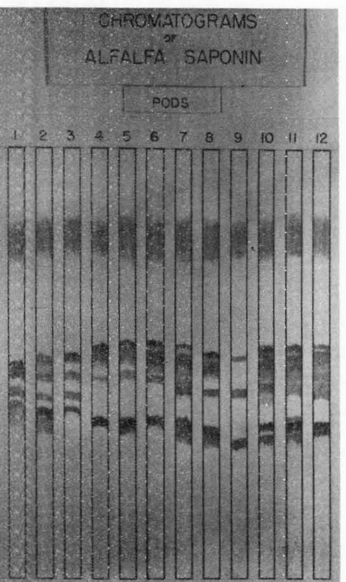


Figure 2. Simulated paper chromatograms of the saponins in the flowers and pods of 12 alfalfa clones

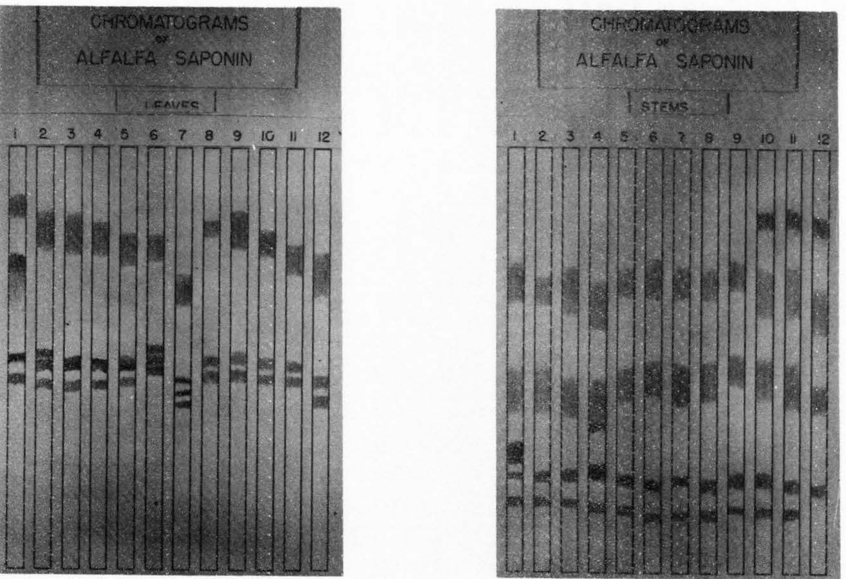


Figure 3. Simulated paper chromatograms of the saponins in the leaves and stems of 12 alfalfa clones

Table 6. Ranked means of the number of curls per pod for 12 clones of alfalfa

Clone number	Rank	Clonal origin	Number of curls per pod		Least significant ranges[a] 5% level Duncan's new multiple range test
12	1	Iran C	2.5		A
8	2	Lahontan	2.3		A B
10	3	Iran A	2.2		A B
2	4	Du Puits	2.2		A B
7	5	Nemastan	2.1		B C
9	6	Afghanistan	1.9		C
11	7	Iran B	1.8		C D
5	8	Vernal	1.8		C D
6	9	Utah 39	1.8		C D
4	10	Teton	1.5		C D
3	11	Iraq	1.5		C D
1	12	Rhizoma	1.2		E
		$\bar{X}$	1.9		
		F value for clones		13.15**	
		Coefficient of variation		.11	

**Significant at the 1 percent level of probability.

[a]Significant difference exists between any two means not found in the same range.

Table 7. Ranked means of the width of the average complete curl for 12 clones of alfalfa

Clone number	Rank	Clonal origin	Width of the average complete curl (in mm)	Least significant ranges[a] 5% level Duncan's new multiple range test
1	1	Rhizoma	6.5	A
4	2	Teton	6.3	A
5	3	Vernal	6.0	B
3	4	Iraq	5.3	C
6	5	Utah 39	5.1	C D
7	6	Nemastan	5.0	C D
2	7	Du Puits	4.8	D E
9	8	Afghanistan	4.7	D E
8	9	Lahontan	4.6	E F
10	10	Iran A	4.4	F
11	11	Iran B	4.4	F
12	12	Iran C	4.3	F
		$\bar{X}$	5.1	
		F value for clones	43.80**	
		Coefficient of variation	.046	

**Significant at the 1 percent level of probability.

[a]Significant difference exists between any two means not found in the same range.

Table 8. Ranked means of the tightness of curls for 12 clones of alfalfa[b]

Clone number	Rank	Clonal origin	Width of the average complete curl (in mm)	Least significant ranges[a] 5% level Duncan's new multiple range test
1	1	Rhizoma	3.4	A
4	2	Teton	2.8	B
5	3	Vernal	2.5	B C
3	4	Iraq	2.4	B C
7	5	Nemastan	2.2	C D
6	6	Utah 39	2.2	C D
9	7	Afghanistan	2.0	D E
2	8	Du Puits	1.8	E F
11	9	Iran B	1.7	E F
8	10	Lahontan	1.6	E F
10	11	Iran A	1.5	F
12	12	Iran C	1.4	F
		$\bar{X}$	2.1	
		F value for clones	17.88**	
		Coefficient of variation	.13	

**Significant at the 1 percent level of probability.

[a]Significant difference exists between any two means not found in the same range.

[b]The appendix contains additional data on tightness of curls.

complete curl, and tightness of the curls. Differences for each morphological characteristic among the 12 clones of alfalfa were highly significant.

Clone 1 was the highest with regard to exposure of vulnerable pod area to chalcid oviposition (Figure 4). A Duncan's New Multiple Range test revealed that pods of clone 1 contain significantly fewer curls than any of the other clones. It also had more space between curls. Hence, clone 1 was the alfalfa which exposed the greatest pod area to attact by the female adult chalcid (i.e., it had the least number of curls per pod and its curls were separated the farthest apart). Clones 3, 4, and 5 also ranked high in regards to the amount of pod area exposed, but they were all significantly lower than clone 1. Clones 8, 10, and 12 were the alfalfas that had the least vulnerable pods.

The curl widths of clones 1 and 4 were significantly larger than the other 10 clones. Clone 5 was significantly smaller than clones 1 and 4 but larger than the remaining nine clones (Figure 5).

Alfalfa Blossom Color

Blossom color rating

A highly significant difference existed among the 12 clones of alfalfa in relation to flower color (Table 9). Flower color was evaluated on a scale ranging from 1 to 10. A value of one described blossoms containing a relative high level of purple or blue pigments whereas a value of 10 represented light-colored blossoms containing no purple or blue pigments.

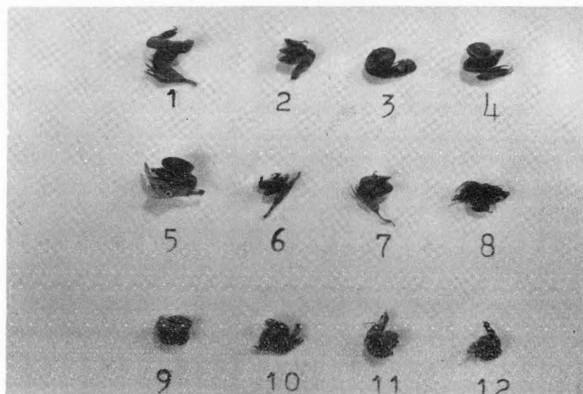


Figure 4. Side view of representive pods from 12 alfalfa clones. (Each pod was taken when it was approximately nine days old.)

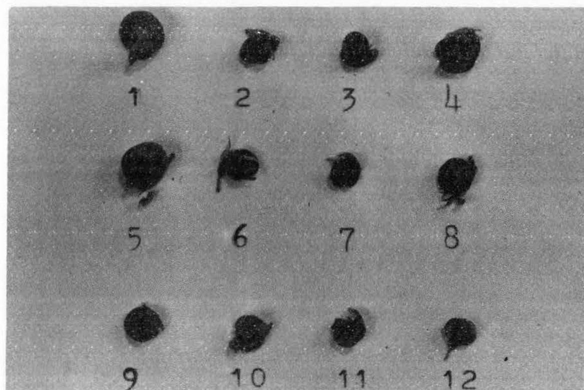


Figure 5. Surface view of representive pods from 12 alfalfa clones. (Each pod was taken when it was approximately nine days old.)

Table 9. Ranked means of the blossom-color rating for 12 clones of alfalfa

Clone number	Rank	Clonal origin	Relative alfalfa blossom color rating	Least significant ranges[a] 5% level Duncan's new multiple range test
5	1	Vernal	9.8	A
1	2	Rhizoma	8.7	B
3	3	Iraq	7.5	C
7	4	Nemastan	6.7	C D
12	5	Iran C	6.5	C D
4	6	Teton	6.5	C D
10	7	Iran A	6.2	D
6	8	Utah 39	5.7	D E
8	9	Lahontan	5.0	E
11	10	Iran B	3.5	F
9	11	Afghanistan	2.7	F G
2	12	Du Puits	2.2	G
		$\bar{X}$	5.9	
		F value for clones	43.36**	
		Coefficient of variation	.14	

**Significant at the 1 percent level of probability.

[a]Significant difference exists between any two means not found in the same range.

Blossom color ratings were made on the 12 clones of alfalfa; the results ranged from 2.2 to 9.8. Clone 5 had the lightest colored flowers followed by clone 1 which was significantly lighter than any of the other 10 clones. Clones 2 and 9 received the lowest color ratings due to a high concentration of dark-colored pigments present in their blossoms.

Blossom preference

The alfalfa seed chalcid showed a preference for the lighter colored alfalfa blossoms over the darker pigmented ones (Figures 6 and 7). A highly significant difference was found in blossom preference among the 12 clones of alfalfa (Table 10).

Table 11 contains a summary of the results of six two-way comparisons involving all possible combinations of four clones of alfalfa. Of the four, clones 1 and 5 had the lightest colored blossoms, whereas clones 2 and 9 had dark-colored blossoms. The flowers of clone 5 were pale yellow with no visible tints of purple or blue pigments while clone 1 had flowers that were pinkish white in color. The flowers of both clones 2 and 9 were dark purple; however, clone 2 and a bluish tint associated with its flowers and clone 9 did not. All six experiments showed a highly significant difference in percent of the total number of chalcids that visited the blossoms of the two particular clones being studied.

Four experiments compared light colored blossoms with dark-colored ones, while the other two experiments involved comparisons of blossoms of similar coloration. The average of the four experiments in percent of

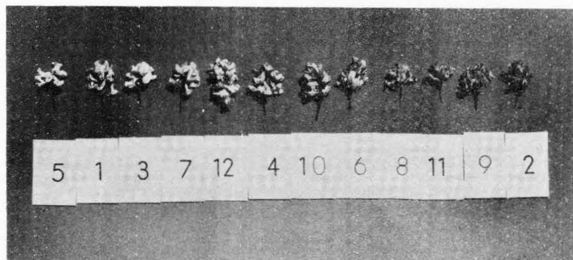


Figure 6. Flowers of 12 clones of alfalfa, arranged from left to right according to the amounts of dark colored pigments present in the flowers

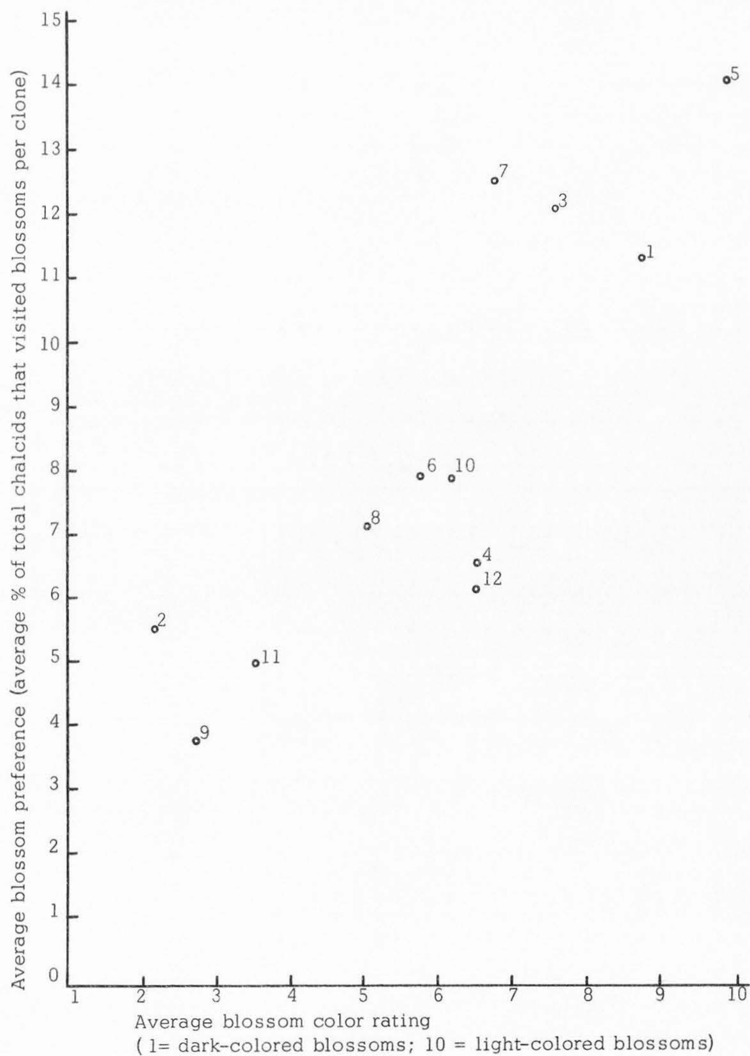


Figure 7. The relationship between blossom color rating and blossom preference for 12 clones of alfalfa

Table 10. Ranked means of the preference showed by alfalfa seed chalcids for the different types of blossoms associated with 12 clones of alfalfa

Clone number	Rank	Clonal origin	Average % of total chalcid that preferred alfalfa blossoms[b]	Least significant ranges[a] 5% level Duncan's new multiple range test
5	1	Vernal	14.1	A
7	2	Nemastan	12.6	A B
3	3	Iraq	12.2	A B C
1	4	Rhizoma	11.3	A B C D
6	5	Utah 39	7.8	C D E
10	6	Iran A	7.8	B E
8	7	Lahontan	7.2	D E
4	8	Teton	6.6	E
12	9	Iran C	6.1	E
2	10	Du Puits	5.5	E
11	11	Iran B	5.0	E
9	12	Afghanistan	3.8	E
		$\bar{X}$	8.3	
		F value for clones	4.77**	
		Coefficient of variation	.41	

**Significant at the 1 percent level of probability.

[a]Significant difference exists between any two means not found in the same range.

[b]Percent chalcids represented the percent of the total number of chalcids which visited the blossoms of all 12 clones combined.

Table 11. Results of six two-way comparisons in blossom preference for all possible combinations of four clones of alfalfa

Comparisons				Percent of total chalcids that preferred alfalfa blossoms per clone				
Clone number	Clonal origin	Color of blossoms	F value for clones	Rep. 1	Rep. 2	Rep. 3	Average	
							Light	Dark
5	Vernal	Light		76.0	73.2	73.7	74.3	
vs.			1589**					
9	Afghanistan	Dark		24.0	26.8	26.3		25.7
5	Vernal	Light		68.5	72.8	64.6	68.6	
vs.			124**					
2	Du Puits	Dark		31.5	27.2	35.4		31.4
1	Rhizoma	Light		66.4	65.6	72.3	68.1	
vs.			147**					
9	Afghanistan	Dark		33.6	34.4	27.7		31.9
1	Rhizoma	Light		74.8	71.8	70.3	72.3	
vs.			568**					
2	Du Puits	Dark		25.2	28.2	29.7		27.7
	Average of light verses dark blossoms----------------------						70.8	29.2
							High	Low
9	Afghanistan	Dark		45.6	46.8	46.6		46.3
vs.			196**					
2	Du Puits	Dark		54.4	53.2	53.4	53.7	
1	Rhizoma	Light		47.5	44.9	44.8		45.7
vs.			47**					
5	Vernal	Light		52.5	55.1	55.2	54.3	
	Average of blossoms of similar coloration---------------------						54.0	46.0

**Significant at the 1 percent level of probability.

chalcids that visited the light flowers was 71 while the average percent of chalcids that visited the dark flowers was 29. However, the experiment comparing light with light-colored blossoms showed a difference in blossom preference of only 54 and 46 percent. In like manner, the experiment comparing dark with dark-colored blossoms also had a visitation difference of 54 and 46 percent.

With all six comparisons, the ratio of the number of chalcids visiting the blossoms of the two particular clones involved were approximately the same as when the experiment was done using all 12 clones combined. For example, the ratio of the comparison of clone 5 against clone 9 was 74 to 26 for the two-way experiment while it was 79 to 21 in the combined experiment. Similarly, the results of the other five two-way comparisons and the results of the experiment where all 12 clones were combined showed no discrepancy in relative blossom preference.

Percent Chalcid Infestation

In 1965, a large population of chalcids were present in the experimental field resulting in a high infestation of alfalfa seed. A highly significant difference in percent chalcid infestation occurred among the 12 clones of alfalfa. The 12 clones showed a wide spread ranging from 74 to 29 percent infested seed. Clone 5 ranked the highest followed closely by clone 4. The seeds of clone 9 were the least susceptible to chalcid infestation being 2.54 times more resistant than clone 5 (Table 12).

Table 12. Ranked means of the 1965 percent chalcid infestation in 12 clones of alfalfa

Clone number	Rank	Clonal origin	Percent chalcid infestation	Least significant ranges[a] 5% level Duncan's new multiple range test
5	1	Vernal	74	A
4	2	Teton	70	A
6	3	Utah 39	63	A B
2	4	Du Puits	57	B C
1	5	Rhizoma	51	C D
11	6	Iran B	46	C D E
8	7	Lahontan	44	D E
7	8	Nemastan	38	E F
3	9	Iraq	38	E F
12	10	Iran C	37	E F
10	11	Iran A	36	E F
9	12	Afghanistan	29	F
		$\bar{X}$	48	
		F value for clones		14.47**
		Coefficient of variation		.16

**Significant at the 1 percent level of probability.

[a]Significant difference exists between any two means not found in the same range.

Relationships Between Variables

Table 13 contains the correlation coefficients of all possible simple (two-way) linear comparisons among the various variables in this study.

Relationships between various characteristics of alfalfa

Correlation coefficients from comparisons between the saponin percentages in the flowers, pods, leaves, and stems of alfalfa were relatively low compared to what one might possibly have predicted. The relationships between saponin in the pods and saponin in the flowers and leaves were particularly low. A negative correlation coefficient occurred between percent saponin in the pods and stems.

The r values for relationships between the three morphological characteristics associated with the pods were the highest among all possible simple comparisons of variables. A negative correlation coefficient appeared between curls per pod and width of the curl and between curls per pod and tightness of curls while a positive correlation coefficient was calculated for width of the curl versus tightness of curls.

A correlation coefficient for alfalfa blossom color versus blossom preference was 0.654.

Relationships between alfalfa characteristics and percent chalcid infestation

Relationships between percent chalcid infestation data for 1965 and each of the previously mentioned characteristics of alfalfa varied from

Table 13. Correlation coefficients of all simple two-way comparisons between nine alfalfa characteristics and percent chalcid infestation, computed from data collected from 12 clones of alfalfa

Alfalfa characteristics and % chalcid infestation	Saponin flowers	Saponin pods	Saponin leaves	Saponin stems	Chalcid infestation
	%	%	%	%	%
% saponin flowers	1.00	.38	.61	.61	.52
% saponin pods	.38	1.00	.21	-.05	.28
% saponin leaves	.61	.21	1.00	.57	.53
% saponin stems	.61	-.05	.57	1.00	.43
% chalcid infestation	.52	.28	.53	.43	1.00
Curls per pod	-.22	-.14	-.56	-.31	-.22
Width of curl	.32	.23	.73	.32	.52
Tightness of curl	.19	.21	.67	.26	.34
Blossom color rating	-.25	.06	.17	-.06	.26
% blossom preference	-.24	.11	.05	-.17	.20

Alfalfa characteristics and % chalcid infestation	Curls per pod	Width of curl	Tightness of curl	Blossom color rating	% blossom preference
% saponin flowers	-.22	.32	.19	-.25	-.24
% saponin pods	-.14	.23	.21	.06	.11
% saponin leaves	-.56	.73	.67	.17	.05
% saponin stems	-.31	.32	.26	-.06	-.17
% chalcid infestation	-.22	.52	.34	.26	.20
Curls per pod	1.00	-.71	-.82	-.30	-.11

Table 13. Continued

Alfalfa characteristics and % chalcid infestation	Curls per pod	Width of curl	Tightness of curl	Blossom color rating	% blossom preference
Width of curl	-.71	1.00	.87	.64	.36
Tightness of curl	-.82	.87	1.00	.56	.36
Blossom color rating	-.30	.64	.56	1.00	.65
% blossom preference	-.11	.36	.36	.65	1.00

an r value of 0.532 for percent saponin in the leaves to an r value of 0.196 for blossom preference.

Saponin content versus chalcid infestation. Correlations coefficients computed for simple linear relationships between percent chalcid infestation and percent saponin in the flowers, pods, leaves, and stems were 0.519, 0.279, 0.533, and 0.429 respectively.

Many theories could be proposed in the attempt to postulate the reason chalcid infestation was positively related to the different saponin percentages of the various clones in this study. Three possible explanations will be offered here: (1) Having soap-like properties, saponin present in the pods may serve as a "lubricant" to facilitate the process of oviposition. (2) Saponin from any or all parts of the plant may act as a token attractant. Dethier (1947, p. 19-20) shows that nature is replete with examples where chemicals serve merely as signposts to attract insects to their host plants for oviposition and/or food. (3) Saponin might

be beneficial to some vital function of the alfalfa seed chalcid in order that it may more successfully complete its normal cycle of metamorphosis. This beneficial effect could occur in all or any one of the stages of the insect's development.

Morphological characteristics versus chalcid infestation. Thomas (1963) measured the number of curls per pod on 17 varieties of alfalfa which had previously been analyzed for relative susceptibility to the alfalfa seed chalcid. Statistical computation showed a close correlation ($r=-0.863$) between varieties selected and the number of curls per pod. Data from this study in 1965 showed a much lower correlation ($r=-0.22$) between these same factors. Four of the varieties sampled by Thomas were among the 12 clones used in this study.

A correlation coefficient from the 1965 study of 0.518 for comparison between width of curl and percent chalcid infestation and of 0.336 between tightness of curls and percent chalcid infestation were obtained.

Theoretically the above mentioned morphological characteristics of alfalfa would act as a mechanical or physical barrier to chalcid damage to alfalfa seed. The more curls per pod and the tighter the curls the less opportunity there would be for chalcid to infest the seeds. The positive correlation between width of curl and percent chalcid infestation would suggest that a wide curl somehow was more conducive to oviposition than a narrow curl. Perhaps this correlation with width of curl was superficial in that the real conducive factors were not curl width but were thickness of the pod tissue (cf. Thomas, 1963, p. 20-22) and/or surface

curviture of the pod. Therefore, these factors would be a function of width of curls.

Alfalfa blossoms versus chalcid infestation. A relatively low correlation coefficient of 0.260 was computed for the relationship between alfalfa blossom color rating and percent chalcid infestation. Similarly an r value of 0.196 was calculated for chalcid blossom preference versus percent infestation.

In relation to both separate variables involving blossom color--viz. blossom color rating and blossom preference, the experiments were not set up like each variable would be in the field during the seasonal time when the chalcids oviposit in the seed (cf. materials and methods). In both experiments a quality determination was estimated for blossoms not taking into account the quantity differences which would be present in the field. Therefore, the real effect of differences in alfalfa blossoms as related to percent chalcid infestation would most likely be the product of quality times quantity of alfalfa blossoms present during the seasonal time when the chalcids oviposit in the seed. Unfortunately no method was employed to ascertain the different clonal quantities of alfalfa blossoms during this critical oviposition period.

Although the results suggest that chalcids prefer to visit lighter colored alfalfa blossoms over darker colored ones, the exact reason why they do is not known at this time. A correlation coefficient of 0.654 between blossom color rating and blossom preference indicates that the color of the blossoms is an important stimulus in determining the relative

attractiveness of each clone. Scent, quality and/or quantity of the nectar, and shape of the different blossoms certainly could have been influential factors also (Von Frisch, 1950).

The importance of blossom preference in relation to chalcid infestation is merely speculation. One supposition is that if chalcids visited the blossoms of a particular clone more than another, then they would tend to oviposit more frequently in the seeds of that clone. Another possible implication of blossom preference comes from studying the feeding habits of adult chalcids in relation to oviposition. Strong (1962) collected adult female chalcids as they emerged from infested seed and caged them individually on single alfalfa racemes. Some of the females were fed a 5 percent honey solution while others were given no food. The results showed a five-fold increase in oviposition from feeding the adult female chalcids. Richardson (1925) indicated that subnormal nutrition, whether due to the quality or quantity of the food, may have a decided effect upon oviposition. Therefore, if blossom preference was related to different qualities or quantities of nectar in alfalfa blossoms, then the end result could very possibly be that more oviposition would occur in clones which contained preferred blossoms.

Percent Chalcid Infestation For 1960 and 1961

Table 14 contains a summary of percent chalcid infestation for the years 1960, 1961 (Rowely, 1962), and 1965. For purposes of comparison, a summarization of the means of the nine alfalfa characteristics analyzed

Table 14. Summary of percent chalcid infestation data for 1960[a], 1961[a], and 1965

Clone number	Clonal origin	Percent chalcid infestation			
		1960	1961	Average of 1960-1961	1965
1	Rhizoma	65	43	54	51
2	Du Puits	62	39	50	57
3	Iraq	61	41	51	38
4	Teton	60	35	48	70
5	Vernal	57	30	44	74
6	Utah 39	52	23	37	63
7	Nemastan	44	28	36	38
8	Lahontan	46	28	37	44
9	Afghanistan	40	21	30	29
10	Iran A	38[b]	19[b]	29[b]	36
11	Iran B	38[b]	19[b]	29[b]	46
12	Iran C	--	--	--	37

[a]Chalcid infestation data for 1960 and 1961 were taken from Rowley's thesis (1962).

[b]No data were available on a clonal basis for Iran A and Iran B; so the varietal percent chalcid infestation for Iran PI 222,178 was substituted for both clones (cf. Table 2).

in this study are listed in Table 15.

Although the amount per clone of infested seeds was lower in 1961, a careful examination of Table 14 reveals that the relative ranking of percent chalcid infestation for 1960 and 1961 was consistent for the two years. However, the relative ranking of chalcid infestation in 1965 deviated somewhat from the data obtained previously. This discrepancy could be accounted for in several ways: (1) The results in 1965 could have been entirely valid because clones were used for infestation determinations whereas in 1960 and 1961 varieties were used. (2) Environmental factors from year to year are usually not consistent and this may have had a decided effect

Table 15. Summary of the data for the nine alfalfa characteristics studied in 1965 and 1966

Clone number	Clonal origin	% saponin flowers	% saponin pods	% saponin leaves	% saponin stems	% saponin average	No. of curls	Curl width	Tightness of curls	Blossom color rating	Blossom preference
1	Rhizoma	2.71	1.97	1.97	.42	1.78	1.2	6.5	3.4	8.7	11.3
2	Du Puits	3.58	1.82	1.64	.45	1.90	2.2	4.8	1.8	2.2	5.5
3	Iraq	2.42	1.28	1.42	.40	1.40	1.5	5.3	2.4	7.5	12.2
4	Teton	3.55	1.23	2.21	.55	1.88	1.5	6.3	2.8	6.5	6.6
5	Vernal	2.23	1.42	1.44	.37	1.39	1.8	6.0	2.5	9.8	14.1
6	Utah 39	2.64	1.44	1.96	.45	1.62	1.8	5.1	2.2	5.7	7.8
7	Nemastan	2.28	1.51	1.48	.25	1.39	2.1	5.0	2.2	6.7	12.6
8	Lahontan	2.43	1.48	1.35	.37	1.41	2.3	4.6	1.6	5.0	7.2
9	Afghanistan	2.19	1.13	1.49	.41	1.30	1.9	4.7	2.0	2.7	3.8
10	Iran A	2.31	1.16	1.16	.36	1.24	2.2	4.4	1.5	6.2	7.8
11	Iran B	2.76	1.69	1.20	.37	1.51	1.8	4.4	1.7	3.5	5.0
12	Iran C	2.17	1.34	1.12	.42	1.26	2.5	4.3	1.4	6.5	6.1

upon the differences and relative ranking in percent chalcid infestation among the 12 alfalfa clones involved. (3) The plants tested for chalcid infestation in 1965 received abnormal cultural practices which possibly might have distorted their normal trend of susceptibility to attact by the alfalfa seed chalcid. Flowers and pods were continually being taken from these plants for future saponin analysis purposes and the proportion removed varied from clone to clone. Six clones namely 1, 3, 8, 10, 11, and 12 necessitated that flowers be taken from them for a longer period of time than the other clones studied. These six clones were the only ones harvested on August 27 through September 1, 1965. This is approximately the same period when the chalcid populations are usually highest in the field.

Of the six only clones 1 and 3 have light colored blossoms. These two clones were also the ones which differed the greatest from the 1960 and 1961 percent chalcid infestation studies (cf. Table 14). In 1965 both clones 1 and 3 were considerably lower in infestation than in 1960 and 1961. Therefore, this could possibly have been due to the reduced level of light colored blossoms during the seasonal time when the chalcids oviposited in the seeds.

Clones 5 and 7 also have light colored blossoms. During the 1965 infestation season clone 5 had a relatively high level of blossoms. Clone 7 blooms earlier than clone 5 and consequently during infestation it had a low level of blossoms. Clone 5 had a very high percent chalcid infestation while clone 7 had a relative low one. Hence, blossom color plus the

quantity of blossoms in the field during infestation could have accounted (at least in part) for the wide difference in infestation associated with these two clones.

SUMMARY AND CONCLUSIONS

The specific purpose of this study was to investigate the possible relationships between chemical composition and/or physical characteristics of alfalfa and its susceptibility to the alfalfa seed chalcid. The major objective was broken down as follows: (1) Determine the bulk saponin content in the flowers, pods, leaves, and stems of some selected alfalfa clones. (2) Measure the number of curls per pod, width of the average complete curl, and tightness of the individual curls of the pods of each clone. (3) Determine if the alfalfa seed chalcid shows a preference for the flowers in full-bloom of one alfalfa clone over another. (4) Determine the percent chalcid infestation of seed from each clone. (5) Calculate the correlation coefficients between these various characteristics of alfalfa and its susceptibility to the alfalfa seed chalcid.

Nine alfalfa characteristics and the determination of the percent alfalfa seed chalcid infestation amounted to 10 variables that were studied. An analysis of variance showed that there were highly significant differences for each variable among the 12 clones of alfalfa that were selected for this study. These significant differences indicate that the alfalfa characteristics could most likely be modified genetically.

The carbon-pyridine procedure was used to determine the saponin content in the flowers, pods, leaves, and stems of the 12 clones of alfalfa. Clones 1, 2, and 6 ranked consistently high in saponin for all

plant parts. Clone 4 contained a large amount of saponin in all parts except in the pods where it ranked tenth. Clones 9, 10, and 12 most frequently had low concentrations of saponin except in leaves of clone 9 which ranked fifth highest in percent saponin.

The simple correlation coefficients for relationships between the saponin percentages of the four plant parts were lower than might have been expected. The percent saponin in the pods particularly showed little relationship to the saponin percentages in the other three plant parts. The correlation coefficients for percent chalcid infestation verus each of the four plant parts were 0.519 for flowers, 0.279 for pods, 0.532 for leaves, and 0.429 for stems.

Three morphological characteristics of the alfalfa pods were examined, namely: number of curls per pod, width of the average complete curl, and tightness of the curls. Clone 1 exposed the greatest pod area to attack by the female adult chalcid (i.e., it had the least number of curls per pod and its curls were separated the farthest apart). Clones 3, 4, and 5 also ranked high in regards to the amount of pod area exposed; but they were all significantly lower than clone 1. Clones 8, 10, and 12 were the clones that had the least vulnerable pods.

Blossom color ratings of the 12 alfalfa clones ranged from 2.2 to 9.8. Clone 5 had the lightest colored flowers followed by clone 1 which was significantly lighter than any of the other 10 clones. Clones 2 and 9 received the lowest color ratings due to the high concentration of dark colored pigments present in their blossoms.

A method was devised to determine if chalcids prefer to visit the blossoms of one alfalfa clone over another. The alfalfa seed chalcids showed a preference for the lighter colored alfalfa blossoms over the darker pigmented ones. A relatively low correlation coefficient of 0.260 was computed for the relationship between alfalfa blossom color rating and percent chalcid infestation. Similarly an r value of 0.196 was calculated for chalcid blossom preference versus percent infestation.

In the late summer of 1965, a large population of chalcids was present in the experimental field resulting in a high infestation of alfalfa seeds. The 12 clones showed a wide spread of percent infested seeds ranging from 74 for clone 5 to 29 for clone 9. The alfalfa plants tested for chalcid infestation in 1965 received abnormal cultural practices which possibly might have distorted their normal trend of susceptibility to attack by the alfalfa seed chalcid. Evidence for this conclusion comes from a wide inconsistency between the correlation coefficient obtained by Thomas (1963) for curls per pod versus percent chalcid infestation and the correlation coefficient obtained in 1965 for the same two variables. Thomas obtained an r value of -0.863 indicating that the more curls per pod the fewer are the number of infested seeds. An r value of only -0.22 was recorded in 1965 for these same two factors.

Because of the lack of confidence in the chalcid infestation data for 1965, the possible relationships between alfalfa characteristics and percent chalcid infestation are not conclusive. Dethier (1947) sums up the complexity of insect resistance when he states that no one stimulus alone

performs the service of guiding an insect to its plant host. The desired end is achieved by a complex array of stimuli working in harmony. Although one "perfect" relationship would be highly desirable, the results from this study and from studies of other researchers (Rowley, 1962; and Thomas, 1963) tend to concur with the statement made by Dethier. Therefore, a multiple correlation, combining some or all of the nine characteristics of this study and most likely other plant and/or environmental variables, appears to be the most feasible approach to the problem.

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APPENDIX

Table 16. Data for tightness of curls obtained by using a calibrated binocular[a]-- 1 unit = 0.8 mm

Replication	Sample number	Clone number 1	2	3	4	5	6	7	8	9	10	11	12
1	1	4.0	2.0	3.5	3.0	2.5	1.5	2.0	2.5	1.5	1.5	2.0	1.8
	2	4.5	2.0	2.5	3.5	2.5	1.5	2.5	1.5	2.5	1.5	2.0	2.0
	3	4.0	2.0	2.0	3.5	2.5	2.0	3.0	2.5	3.0	2.0	1.5	1.5
	4	4.0	2.0	4.0	4.0	2.3	2.5	1.5	2.5	1.0	1.8	2.0	2.0
	5	4.0	3.0	2.0	3.0	3.0	3.0	3.5	2.0	4.0	2.0	1.5	1.5
2	6	4.0	2.5	2.5	1.5	3.0	3.0	3.5	1.5	1.5	1.8	2.0	2.0
	7	5.0	2.0	3.0	3.0	4.0	2.5	4.0	1.0	2.3	1.5	1.5	1.5
	8	4.0	1.5	4.5	4.0	2.0	4.0	2.0	2.0	2.0	1.5	1.8	1.8
	9	4.0	3.5	4.0	3.5	4.0	3.0	3.5	2.5	2.0	1.8	1.5	1.5
	10	4.0	2.5	4.0	4.0	3.0	2.5	2.5	2.0	2.3	1.5	1.5	1.5
3	11	4.0	2.0	4.5	4.0	2.0	4.0	2.5	2.0	3.0	1.5	2.5	1.5
	12	4.0	1.8	3.0	5.0	4.0	2.5	2.5	1.5	1.8	1.8	2.0	1.5
	13	5.0	1.5	4.0	4.0	3.0	3.5	4.0	1.5	2.5	2.0	2.0	2.0
	14	4.0	2.0	2.0	2.0	3.0	2.5	4.0	2.5	4.0	4.0	1.5	1.5

Table 16. Continued

Replication	Sample number	Clone number 1	2	3	4	5	6	7	8	9	10	11	12
	15	4.0	2.0	2.5	4.0	4.0	2.5	3.0	2.0	4.0	1.5	4.0	2.3
4	16	4.0	2.0	3.0	3.0	4.0	3.0	1.5	3.0	3.0	1.8	4.0	1.5
	17	4.0	2.5	4.0	3.5	3.5	1.5	2.5	2.5	2.3	2.3	1.5	2.0
	18	4.0	2.0	2.0	3.5	4.0	2.5	2.5	1.3	2.0	1.5	1.5	1.8
	19	4.0	2.0	2.0	4.0	3.0	3.5	2.0	2.5	3.0	2.0	2.0	2.0
	20	5.5	2.5	2.0	4.0	3.0	3.5	2.5	1.5	2.0	1.8	4.0	2.0
Average		4.2	2.2	3.1	3.5	3.1	2.7	2.8	2.0	2.5	1.9	2.1	1.8

[a] All pods containing one or less than one curl were given the value of 4.0 as an arbitrary measure of curl tightness. These values were underlined to designate them from the other measurements. (The maturity of the sampled pods was approximately 7 to 11 days from fertilization.)

Table 17. Saponin determination data for flowers

Replication	Description	Clone number 1	2	3	4	5	6
1	% CAP saponin	4.41	5.45	3.89	5.12	3.30	3.92
	% Purity	63.4	65.4	60.4	69.1	70.4	69.5
	% Pure saponin	2.80	3.56	2.35	3.54	2.32	2.72
2	% CAP saponin	3.91	5.63	3.62	5.33	3.64	4.21
	% Purity	69.1	67.5	68.8	66.0	64.0	61.4
	% Pure saponin	2.70	3.80	2.49	3.52	2.33	2.58
3	% CAP saponin	4.18	5.44	3.61	5.58	3.85	4.27
	% Purity	65.4	65.0	69.5	64.4	54.3	61.3
	% Pure saponin	2.73	3.54	2.51	3.59	2.09	2.62
4	% CAP saponin	4.31	5.41	4.22	----	3.77	----
	% Purity	60.5	63.4	58.2	----	58.1	----
	% Pure saponin	2.61	3.43	2.46	----	2.19	----
5	% CAP saponin	----	----	3.99	----	----	----
	% Purity	----	----	57.3	----	----	----
	% Pure saponin	----	----	2.29	----	----	----
Average of % pure saponin		2.71	3.58	2.42	3.55	2.23	2.64

Replication	Description	Clone number 7	8	9	10	11	12
1	% CAP saponin	3.73	3.57	3.67	3.71	4.33	3.58
	% Purity	64.3	69.9	61.2	62.5	63.7	60.9
	% Pure saponin	2.40	2.50	2.25	2.32	2.76	2.18
2	% CAP saponin	3.66	3.82	3.56	3.59	4.27	3.33
	% Purity	59.6	64.3	61.3	65.3	64.0	66.7
	% Pure saponin	2.18	2.46	2.18	2.34	2.73	2.22
3	% CAP saponin	3.69	3.58	3.34	3.63	4.35	3.82
	% Purity	61.6	65.1	63.8	62.9	64.3	55.3
	% Pure saponin	2.27	2.33	2.13	2.28	2.80	2.11
4	% CAP saponin	----	----	----	----	----	----
	% Purity	----	----	----	----	----	----
	% Pure saponin	----	----	----	----	----	----
5	% CAP saponin	----	----	----	----	----	----
	% Purity	----	----	----	----	----	----
	% Pure saponin	----	----	----	----	----	----
Average of % pure saponin		2.28	2.43	2.19	2.31	2.76	2.17

Table 18. Saponin determination data for pods

Repli-cation	Description	Clone number 1	2	3	4	5	6
1	% CAP saponin	2.88	2.79	2.00	1.79	2.18	2.42
	% Purity	69.0	65.4	66.3	70.9	70.8	62.5
	% Pure saponin	1.99	1.82	1.33	1.27	1.54	1.51
2	% CAP saponin	2.66	2.65	2.02	1.76	2.05	2.11
	% Purity	71.7	71.1	67.3	70.2	69.5	69.2
	% Pure saponin	1.91	1.88	1.36	1.24	1.42	1.46
3	% CAP saponin	2.92	2.67	1.81	1.93	2.15	1.98
	% Purity	69.1	68.8	68.6	60.6	69.2	68.3
	% Pure saponin	2.02	1.84	1.24	1.17	1.49	1.35
4	% CAP saponin	----	2.67	1.81	----	----	2.31
	% Purity	----	65.6	64.6	----	----	62.4
	% Pure saponin	----	1.75	1.17	----	----	1.44
Average of % pure saponin		1.97	1.82	1.28	1.23	1.42	1.44

Repli-cation	Description	Clone number 7	8	9	10	11	12
1	% CAP saponin	2.34	2.21	1.74	1.94	2.50	1.95
	% Purity	67.4	65.6	61.5	59.0	67.9	69.4
	% Pure saponin	1.58	1.45	1.07	1.14	1.70	1.35
2	% CAP saponin	2.28	2.36	1.91	2.04	2.70	2.07
	% Purity	67.2	62.8	61.6	59.1	62.3	62.8
	% Pure saponin	1.53	1.48	1.18	1.20	1.68	1.30
3	% CAP saponin	2.17	2.31	1.81	1.93	2.63	2.23
	% Purity	65.5	65.9	62.3	59.6	64.4	60.8
	% Pure saponin	1.42	1.52	1.13	1.15	1.69	1.36
4	% CAP saponin	----	----	----	----	----	----
	% Purity	----	----	----	----	----	----
	% Pure saponin	----	----	----	----	----	----
Average of % pure saponin		1.51	1.48	1.13	1.16	1.69	1.34

Table 19. Saponin determination data for leaves

Repli-cation	Description	Clone number 1	2	3	4	5	6
1	% CAP saponin	3.17	2.85	2.46	3.68	2.34	3.57
	% Purity	64.0	58.0	58.4	61.2	58.2	56.4
	% Pure saponin	2.03	1.65	1.44	2.25	1.36	2.01
2	% CAP saponin	2.85	2.63	2.35	3.55	2.24	3.03
	% Purity	66.7	61.0	57.5	60.0	62.7	65.2
	% Pure saponin	1.90	1.60	1.35	2.13	1.40	1.98
3	% CAP saponin	3.12	2.82	2.40	3.61	2.42	3.23
	% Purity	63.2	62.8	65.8	62.2	64.4	58.7
	% Pure saponin	1.97	1.77	1.58	2.25	1.56	1.90
4	% CAP saponin	----	2.55	2.23	----	2.02	----
	% Purity	----	60.8	58.5	----	60.8	----
	% Pure saponin	----	1.55	1.30	----	1.23	----
Average of % pure saponin		1.97	1.64	1.42	2.21	1.44	1.96

		Clone number 7	8	9	10	11	12
1	% CAP saponin	2.53	2.20	2.51	2.04	2.25	1.89
	% Purity	57.2	60.6	58.2	55.1	54.5	60.0
	% Pure saponin	1.45	1.33	1.46	1.12	1.23	1.13
2	% CAP saponin	2.55	2.34	2.61	2.11	2.04	1.97
	% Purity	56.6	56.7	58.8	55.6	57.1	55.0
	% Pure saponin	1.44	1.33	1.53	1.17	1.16	1.08
3	% CAP saponin	2.70	2.57	2.65	2.00	2.19	2.06
	% Purity	57.8	53.7	55.6	53.2	54.9	55.7
	% Pure saponin	1.56	1.38	1.47	1.06	1.20	1.15
4	% CAP saponin	----	----	----	2.27	----	----
	% Purity	----	----	----	56.0	----	----
	% Pure saponin	----	----	----	1.27	----	----
Average of % pure saponin		1.48	1.35	1.49	1.16	1.20	1.12

Table 20. Saponin determination data for stems

Replication	Description	Clone number 1	2	3	4	5	6
1	% CAP saponin	0.87	0.73	0.68	1.01	0.69	0.76
	% Purity	43.8	60.0	54.7	53.5	57.9	54.8
	% Pure saponin	0.38	0.44	0.37	0.54	0.40	0.42
2	% CAP saponin	0.69	0.81	0.81	0.95	0.68	0.80
	% Purity	57.6	61.6	56.2	58.8	52.9	55.4
	% Pure saponin	0.40	0.50	0.46	0 56	0.36	0.44
3	% CAP saponin	0.85	0.76	0.69	0.94	0.73	0.88
	% Purity	58.2	54.5	52.8	59.8	50.0	55.2
	% Pure saponin	0.49	0.41	0.36	0.56	0.36	0.49
Average of % pure saponin		0.42	0.45	0.40	0.55	0.37	0.45

Replication	Description	Clone number 7	8	9	10	11	12
1	% CAP saponin	0.49	0.70	0.74	0.78	0.77	0.73
	% Purity	44.4	50.0	51.9	43.9	45.1	54.7
	% Pure saponin	0.22	0.35	0.38	0.34	0.35	0.40
2	% CAP saponin	0.60	0.81	0.75	0.90	0.74	0.77
	% Purity	48.3	45.2	56.4	40.0	50.0	54.5
	% Pure saponin	0.29	0.37	0.42	0.36	0.37	0.42
3	% CAP saponin	0.52	0.69	0.79	0.71	0.72	0.73
	% Purity	47.8	56.4	52.9	53.4	52.1	59.0
	% Pure saponin	0.25	0.39	0.42	0.38	0.38	0.43
Average of % pure saponin		0.25	0.37	0.41	0.36	0.37	0.42