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1955
DETECTION OF COUMARIN IN SEEDS INVOLVING CROSSES
BETWEEN TWO SPECIES OF MELILOTUS

by

William H. Davis

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

of

MASTER OF SCIENCE

in

Agronomy

UTAH STATE AGRICULTURAL COLLEGE
Logan, Utah

1955

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I am deeply grateful to Dr. DeVere R. McAllister for suggestions and material aid which led to the selection and completion of the problem. My thanks are also due Dr. George W. Cochran for the use of laboratory equipment in the experiment.

William H. Davis

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WESTERN BOND

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SECRET

WESTERN BOND

INTRODUCTION

Sweetclover has become increasingly important as a forage and green manure crop. The main objection in recent years to sweetclover is its coumarin content, identified by a sweet odor and bitter taste, making it less desirable to farm animals. When sweetclover hay spoils, the coumarin is converted into dicoumarol which is toxic to animals, especially to ruminants. When ingested this may cause internal and/or external hemorrhages.

Successful crosses between Melilotus alba (biennial white sweetclover) and Melilotus dentata (Banat sweetclover) have been made by Dr. W. K. Smith, University of Wisconsin, creating a hybrid possessing the low coumarin factor of Banat sweetclover. Seeds from these low coumarin hybrids have been released to plant breeders in the United States and Canada, but the important problem of breeding for local conditions and other desirable agronomic characteristics (See Figures 4, 5, and 6) still remains.

One criterion for success of new varieties to be produced will be the retention of the low coumarin factor. Several tissue tests have been devised for use by plant breeders. Slatensek and Washburn (1944) devised a rapid fluorometric method which is quite satisfactory for detecting coumarin in tissue, but plants still have to be grown

before the test can be made. This necessitates carrying many plants containing genes for high coumarin. If an adequate seed test for coumarin could be provided, it would greatly speed up a breeding program. If, for example, a low coumarin annual sweetlover was the goal of a plant breeding program, it would be possible to eliminate approximately three-fourths of the seed produced from a cross of the F_1 's resulting from an annual high coumarin plant and a biennial low coumarin plant on the coumarin factor alone (only one factor is involved). This would reduce the amount of greenhouse space needed and the need of transplanting unwanted seedlings into the field. Considerable saving of time and money could be effected by proper use of an adequate seed test. However, no rapid and reliable seed test for coumarin has yet been devised. Roberts and Link (1937) reported a micromethod of detecting coumarin in seeds and green tissue. It was slow and difficult to perform under ordinary conditions and destroyed the seeds in the testing process. Their test did explain that coumarin in the seed is usually held in bound form, *i.e.*, tied up in glucosidic compounds within the seed. It takes one hour and a minimum temperature of 40 degrees C. to release the bound coumarin (Roberts and Link, 1937). Heating the tissue in strong alkali solutions for 1.5 hours will also release most of the bound coumarin in plant tissue.

The objective of this research is to devise a method of testing seed for coumarin content which is simple, rapid,

inexpensive and reliable; yet will leave the seed viable after sampling. The present methods of seed testing will be evaluated and modified to obtain the objective. The seed test that is selected will be checked for accuracy by growing the seeds, then testing the tissue of the plants produced therefrom.

REVIEW OF LITERATURE

Sweetlover occupies a prominent place in American and European agriculture despite a few obvious drawbacks. Outstanding among these is the presence of coumarin, which causes sweetlover to be less palatable to animals. Sweetlover also causes bleeding in cattle and sheep, especially when they are injured or operated on after eating this forage. When fed spoiled sweetlover hay, bleeding may be so severe as to cause death from either internal or external hemorrhaging. This condition has been called "Sweetlover disease" or the "Bleeding disease". Other names may exist in other localities. Stevenson and White (1940) reported that coumarin is also responsible for "Melilot taint" of wheat in Canada which has caused thousands of bushels of wheat to be rejected for use as flour.

Coumarin is used extensively in the perfume industry because of its sweet odor. Formerly extracted from "Tonka beans", it is now produced synthetically from salicylaldehyde, sodium acetate and acetic anhydride by the Perkins reaction. Coumarin shows little reactivity with acids, but reacts with most basic ions to form coumarin salts.

Roberts and Link (1937) first isolated coumarin in sweetlover. In 1941, Campbell and Link reported the isolation and crystallization of dicoumarol from spoiled

sweetclover hay. They isolated the compound from experimentally produced spoiled hay as well as from hay that had actually killed cattle. Diccumarol is the result of two coumarin molecules uniting through microbial activity. Used extensively in medicine and veterinary practices to reduce the clotting power of the blood, the compound is also used as an effective rat poison. Gilgoly (1952) reports that rats are unable to detect the presence of dicoumarol in the poisoning agent "Warfarin" (formerly W. R. F. N. 42), a trade name for a dicoumarol product developed at the University of Wisconsin by Link (1950).

The reaction of cattle and sheep to coumarin and dicoumarol is quite similar, but it takes considerably less dicoumarol to get the same reaction in the animals. Approximately fifty grams of spoiled sweetclover hay contains 1.5 grams of dicoumarol which is sufficient to cause "Sweetclover disease". Removing the animals from sweetclover pasture and hay will relieve the symptoms of the disease.

Dicoumarol changes the ability of blood capillaries to retain the blood and also increases the prothrombin time. In Germany Kuschinsky and Ludwig (1950) by injecting dicoumarol into mice reported that during the first seven hours a definite increase of blood-vessel permeability was noted. After eleven hours the prothrombin time was almost always increased while permeability in most cases was no longer high. They concluded that dicoumarol has a rapid effect on blood-vessel permeability unrelated to its effects on

prothrombin time. Injection of vitamin K is an effective control measure for bleeding animals. Recovery may be effected by intravenous injections of defibrinated blood (500 to 1000 cc) of normal bovines. This may cure animals even after they are down. In some cases it may be practical to give a blood transfusion directly to the sick animal. In all cases removal from sweetlover hay and pasture is essential. Ruminants are far more susceptible than non-ruminants to the effects of dicoumarol. Non-ruminants digest their food in a reverse manner to ruminants and the effects of coumarin and dicoumarol are not felt so severely.

While chemists and veterinarians were having some success with chemical isolation and control methods, plant breeders were not quite so fortunate at an early date. Many investigators reported that some species of low coumarin sweetlover existed and were possible sources of breeding stock. However, almost all attempts to hybridize species of sweetlover met with little success. Natural crosses between Melilotus alba and Melilotus officinalis had been observed. Kirk (1929) obtained one natural cross from 11,400 crosses. He planted a row of white-flowered sweetlover and surrounded them with 10 varieties or species of yellow-flowered sweetlover. The one F_1 hybrid had intermediate flower color. Greenhouse experiments by Stevenson and Kirk (1934) failed to produce any viable seed from 9,000 crosses between M. alba and M. officinalis. Crosses between M. alba and Melilotus sauveolens were quite successful and some F_1

hybrids were grown. They observed that pollination and fertilization took place when species of Melilotus were crossed but in a short time the embryo aborted. Apparently the embryo was incompatible with the maternal tissue surrounding it. By removing the immature embryos from the maternal tissue and culturing in nutrient agar solution, they were able to prolong embryo life. First offered as a possible method of obtaining successful interspecific crosses in Melilotus, this later became an important technique in obtaining low coumarin yellow sweetclovers by interspecific crosses (Webster, 1954). At the same time that interspecific crosses were being made, they tried intergeneric crosses between Melilotus and Medicago, Melilotus and Trigonella. None of these crosses produced any results applicable to a sweetclover breeding program.

Schroek (1948) in Germany spent several years looking for mutant types of sweetclover with a low or free coumarin factor. He reported that two species of sweetclover, Melilotus dentata (Danat sweetclover) and Melilotus messanensis were low in coumarin. However, they were of little agronomic value and produced chlorophyll deficient hybrids when crossed with M. alba (biennial white sweetclover) or M. officinalis (yellow sweetclover). These hybrids lived only six to fourteen days. Schroek continued his investigations for a low coumarin sweetclover, testing hundreds of thousands of seeds with a microtechnique. In this method, a slice of cotyledons from each seed was mounted

on a microscope slide and bathed in ammonia vapor for a short time. After removal it was tested for fluorescence under a fluorescent microscope. The presence of a greenish-yellow fluorescence indicated coumarin, while a faint or lack of fluorescence indicated low or no coumarin. All his results in this program were destroyed by bombing during World War II.

In Canada, Stevenson and White (1940) investigated numerous species of sweetclover, looking for low coumarin stock. They found a great deal of variation in the coumarin content of sweetclover. Some varieties and species varied greatly with the amount of coumarin held in a bound form. White and Homer (1940) then attempted to select for a low coumarin strain using strains of biennial white sweetclover as the source of variation. They found a strain which had a low coumarin test, but later it was discovered that the coumarin was held in a bound form. (This is the first reported case of bound coumarin in the plant.) The strain was actually high in coumarin when tested by a modified fluorometric test (White and others, 1952).

In the United States, Roberts and Link (1937) reported a rapid colorimetric method for testing seed and green tissue of Helilotus spp. for coumarin. They determined that seed contained coumarin, usually a little higher percentage than the plants, and that it was held mostly in a bound form. From their conclusions it was determined that all previous seed tests based on alcoholic or steam extraction

methods were inaccurate (Clayton and Larmour, 1935; Duncan and Dustman, 1937). Previous tests had failed to consider the bound coumarin in seeds or plant tissue. According to Campbell and Link (1937), it takes one hour at 40° C. for the enzymatic release of bound coumarin prior to extraction. Later methods (Slatensek and Washburn, 1944; White et al., 1952) eliminated this enzymatic release period by using strong alkali solutions to effect coumarin release in plant tissue.

In 1943, W. K. Smith crossed M. dentata with M. alba and found the usual chlorophyll deficient hybrids that others had obtained. He then cleft-grafted the F₁ hybrid seedlings onto mature one year old plants of M. officinalis and M. alba. Plants were first prepared by cutting top growth back and potting the plants from the field toward the end of the first season of growth. They were then held in a slowly-growing condition at a low temperature and after a few days were brought into a warm greenhouse. The shoots were allowed to grow approximately six inches, then the distal one-quarter of a shoot was removed just above a node and discarded. The remaining portion of the stem was split longitudinally about half an inch and the pith of one half gouged out. The seedling to be grafted was dug, cut at the base of the hypocotyl and a thin slice removed from each side leaving the hypocotyl blunt and wedge-shaped. The seedling was then inserted into the cleft of the plant, matching cambiums. The stems were tied with a soft thread

to maintain the contact. All grafts were shaded until the scions were established. A few of the grafts were successful with the defective seedling producing chlorophyll and continuing leaf, stem and flower development. The F_1 hybrids were self sterile, but seeds were produced by backcrossing the hybrid with M. alba using alba as the pollen plant.

This pioneer cross is the source of all low coumarin sweetclover now used for plant breeding in the United States and Canada. Once the dominant gene for high coumarin was eliminated, it became easy to maintain genetical purity because the hybrid does not cross readily in the field with other species of sweetclover. Some genetical incompatibilities are still found within the hybrids and some are drastic enough to cause death of the plant.

Homer and White (1941) and Schrock (1948) working on the genetics of coumarin reported that high coumarin was inherited as a single dominant factor. In 1948, W. K. Smith revised the concept of the genetical inheritance of coumarin. His results indicate that coumarin is inherited through two factors. One gene shows high coumarin dominant over low coumarin. In absence of this genetical factor the coumarin content is reduced from approximately 2.5 percent to 0.5 percent (influenced by environment). The other gene appears to have no dominant allele and reduces the coumarin content to 0.1 percent. The interaction of these two genetical factors make M. dentata comparatively free of

coumarin. However, the seeds of M. dentata are known to contain some coumarin. No sweetclover varieties or species are known to be free of both genetical factors. It is not important from the plant breeding point of view, however, because removal of the high coumarin gene is sufficient to make sweetclover safe and palatable to animals. Coumarin is considered sufficiently low when not more than 0.5 percent is found in green plants.

Webster (1954) reported that he has been able to develop a low coumarin yellow sweetclover by crossing Smith's low coumarin strains with M. officinalis. The embryo is removed from the maternal tissue and growth continued by culturing in sterile nutrient solutions. This removed the maternal tissue which caused the embryo to abort. He hopes to be able to release a few of these crosses to other plant breeders in the near future.

Many of these new low coumarin releases will entail further crossing with other local high coumarin varieties to gain certain agronomic characteristics; i.e., annual habit, disease resistance, etc.

A seed test for coumarin will be devised to reduce the necessity of growing and maintaining the numerous progeny of the F_1 selfs and crosses. Such a test would eliminate approximately three-fourths of the seeds tested on the coumarin factor alone (only one factor is involved). The sample taken from each seed would have to be small enough to allow them to germinate and develop normally. Only a

small coumarin-bearing sample is needed when treated with NaOH to form a fluorescing sodium salt of coumarin. A small portion of the cotyledons from each seed could be removed and tested, leaving each seed viable for planting.

However, no tests have determined if it is possible to predict accurately the coumarin inheritance of a plant from an individual seed test. Also to what extent such individual seed tests will correlate with plant tissue tests for coumarin is not definitely known. To answer these questions an experiment based on individual seed tests for determining coumarin content will be carried out.

METHODS OF PROCEDURE

Method I

The first method attempted for the seed tests was that outlined by Schrock (1948) in Germany. He used a microscope technique. A slice of the cotyledons of each seed was treated with ammonia vapor for a short time and observed under a fluorescent microscope. The seeds that contained coumarin fluoresced a bright greenish-yellow, those of no or little coumarin showed no or little color. However, when an attempt was made to repeat this test with seeds of known low and high coumarin inheritance, it proved difficult to note differences. All seed samples fluoresced but the quantity of fluorescence was difficult to measure or observe. His article gave no time limit for bathing the samples in ammonia steam and did not indicate the type of light used on the microscope.

Method II

The next method was based on the same principle as the test of Slatensek and Washburn (1944). Their test was one in which a sodium salt of coumarin was formed by heating a specified amount of NaOH with coumarin-containing tissue of sweetclover. The plant tissue could then be rated by reading the samples under ultra violet light, where a greenish-yellow fluorescence indicates coumarin or a red color indicates low coumarin. The samples could also be rated

quantitatively by use of a Coleman fluorometer and a set of standards.

This test was modified so that seeds could be tested and their coumarin content determined without destruction of the seeds. There was, however, no chlorophyll in the seeds to give the contrasting colors as were obtained in the tissue test. Another feature of seeds is that they all contain some coumarin. This necessitates the addition of chlorophyll in some form to overcome the lack of color variance in the seed test.

The test was set up to utilize only a small part of each seed and then allow the balance to develop into a mature plant. Thus, it was hoped that we could determine the coumarin content of each seed and keep only those of known low coumarin for a breeding program.

Procedure

One thousand seeds were chosen from two sources. Three hundred and thirty seeds were selected from a strain of Melilotus alba annua, called "Floranna", which has known high coumarin content, and seven hundred and seventy seeds represented crosses of Dr. W. K. Smith. The latter seeds represented the F_3 generation of the original M. alba and M. dentata cross. Some contained the low coumarin and some the medium coumarin factor.

All the seeds were placed in a jar, shaken thoroughly and each seed drawn at random from the whole. The seeds were too hard to cut directly, necessitating softening them

with water. A square refrigerator dish was placed upside down in a pan of water. A paper towel soaked in water was placed over the dish with a section trailing in the water to maintain a source of moisture for the seeds placed between two large filter paper pads on the towel. Three to five hours at room temperature (26° C.) was sufficient time to start release of the bound coumarin in the seeds and to soften seed coat for cutting. If it became necessary to stop cutting, the trailing section of the towel was removed from the water. Drying of the seeds seemed to have little effect on subsequent swelling and germination. It was discovered later that many of the seeds remained hard even in the presence of moisture. This made it necessary to scarify approximately half of the seeds by placing them in concentrated sulfuric acid for three minutes, draining the acid off, and washing the seeds with water. This caused most of the remaining seeds to swell. It was best not to let the seed coat become ruptured before cutting the cotyledons. If ruptured seeds were found, it became necessary to dissect those seeds immediately or throw them away. Even with scarification a few seeds failed to swell, making it necessary to add two hundred more seeds to the experiment from the original sources.

To facilitate cutting of the seeds, they were placed under a portable hand lens supplied with a light. Using a scalpel each seed was dissected, removing the tips of the cotyledons at approximately the same place. The seed coat,

representing maternal tissue, was removed at this time so that the test would indicate only the coumarin content of the embryo. The slices of cotyledons were placed in numbered test tubes. The remaining portions of the seeds were transferred to a soil spot plate which was numbered in a corresponding manner. The spot plate had been prepared by filling each spot with water and adding a piece of filter paper to buoy up the seed. After filling each spot plate with dissected seed, it was covered with a moist paper towel to prevent the seeds from drying out and kept in a moist warm place for at least one day before planting.

Two ml. of 2.5 N NaOH and three mg. of air-dried alfalfa leaf tissue were added to the seed samples in the test tubes. (Earlier tests had been tried without adding alfalfa leaves for a source of chlorophyll but difficulty was encountered trying to read the results.) The test tubes were then placed in racks which were put into a controlled water bath for 1.5 hours at a temperature of 94° C. After removal from the water bath, the samples were taken into a dark room for reading under a Burton ultra violet black light. (A lamp sealed against visible light aids accuracy in reading.) The samples were set in the direct rays of the lamp for at least five minutes before reading. When a sample fluoresced a bright greenish-yellow, a rating of high coumarin was marked against the seed number; if the sample showed a red fluorescence, a mark of low coumarin was put by the number. Any sample showing intermediate colors was

given a definite mark of either low or high coumarin by a comparative method. Later low coumarin plus and high coumarin minus ratings were given. Only two definite categories were maintained for statistical analysis.

After each seed had been numbered and germinated on the spot plate, it was planted in especially prepared flats with paper enclosed soil sections. One seed was placed to a section, each flat containing 540 sections. The seeds were covered with vermiculite and sand, then watered as often as was observed to be necessary.

In about eight weeks (See Figure 7) the young seedlings had reached sufficient size to be sampled. Not all the seedlings survived, as damping off and other causes reduced the number to 710 plants. From each seedling a sample of three or four trifoliolate leaves was removed, air-dried and weighed. A twenty mg. sample was tested by using a modified, rapid fluorometric method (White et al., 1952). The results of the two tests were then compared, using the plant tissue test as the standard.

RESULTS

Chi-square analysis of seed and plant tissue tests

| No. of Plants | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | Chi-square |
|---------------|-----|----|----|-----|-----|-----|-----|----|----------------|
| 65 | 32 | 14 | 18 | 21 | 27 | 19 | 23 | 16 | 4.9 * |
| 100 | 49 | 8 | 1 | 42 | 28 | 26 | 22 | 22 | 68.5 ** |
| 100 | 49 | 4 | 2 | 45 | 27 | 26 | 24 | 23 | 77.7 ** |
| 100 | 67 | 2 | 3 | 28 | 48 | 21 | 22 | 9 | 81.2 ** |
| 100 | 58 | 1 | 6 | 35 | 38 | 21 | 26 | 15 | 71.6 ** |
| 100 | 62 | 4 | 2 | 32 | 42 | 24 | 22 | 12 | 70.7 ** |
| 100 | 62 | 8 | 3 | 27 | 46 | 24 | 20 | 10 | 56.9 ** |
| 25 | 9 | 0 | 0 | 16 | 5 | 6 | 6 | 10 | 25.0 ** |
| 625 | 356 | 27 | 17 | 225 | 229 | 154 | 144 | 98 | 451.7** Totals |

1 d.f. at P = .90
Chi-square = .016

1. Low coumarin, plants and seeds.
2. Low coumarin plants; high coumarin seeds.
3. High coumarin plants; low coumarin seeds.
4. High coumarin, plants and seeds.
5. Calculated value for column 1.
6. Calculated value for column 2.
7. Calculated value for column 4.
8. Calculated value for column 3.

* This chi-square value is significant but was not included in the totals because of faulty technique.

** These values are highly significant and indicate that the relationship between the two tests is closely correlated.

DISCUSSION AND CONCLUSIONS

The experiment was conducted, comparing a new seed test for coumarin against a rapid plant tissue method. In the tests it proved to be a disadvantage to separate the seeds into two categories by visually predicting either high or low coumarin. The seeds registering a vivid greenish-yellow or red fluorescence were easy to distinguish between, but the intermediate group was hard to separate with accuracy (See Figure 1). Any future tests should include at least a third, or intermediate, classification.

Before the test was started, correspondence with Mr. J. E. R. Greenshields, Canadian Department of Agriculture (1954), suggested that I try adding a given amount of chlorophyll to each seed sample. This suggestion aided greatly. Without chlorophyll, all seeds indicated some greenish-yellow fluorescence and the only way to rate them was by comparison. An all-green color range proved to be difficult to read (See Figure 3). Chlorophyll obscures the light green colors and gives a contrasting red color which aids greatly in reading samples. Doubling the amount of chlorophyll (See Figure 2) seemed to have little effect on just how much coumarin could be masked. Trial and error procedure later indicated that approximately eight mg. was an adequate amount of alfalfa leaf to add for good vivid



Figure 1. Seed test with added chlorophyll showing fluorescent colors developed under ultra violet light; low coumarin on the left, intermediate in the middle and high on the right. The colors on the right appear bright greenish-yellow rather than the blue in the photograph.

Printon print (14 seconds at 5 m.c.) from an Anso Color transparency (F 4 at 7 minutes).

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Figure 2. Fluorescent colors developed when testing the coumarin content of sweetclover seeds showing the effect of adding varying amounts of chlorophyll in the form of dried alfalfa leaves. In each series of three test tubes, that on the right received no chlorophyll, the center received 3 mg. and that on the left received 6 mg.

Printon print (14 seconds at 5 m.c.) from an Anso Color transparency (F 4 at 7 minutes).

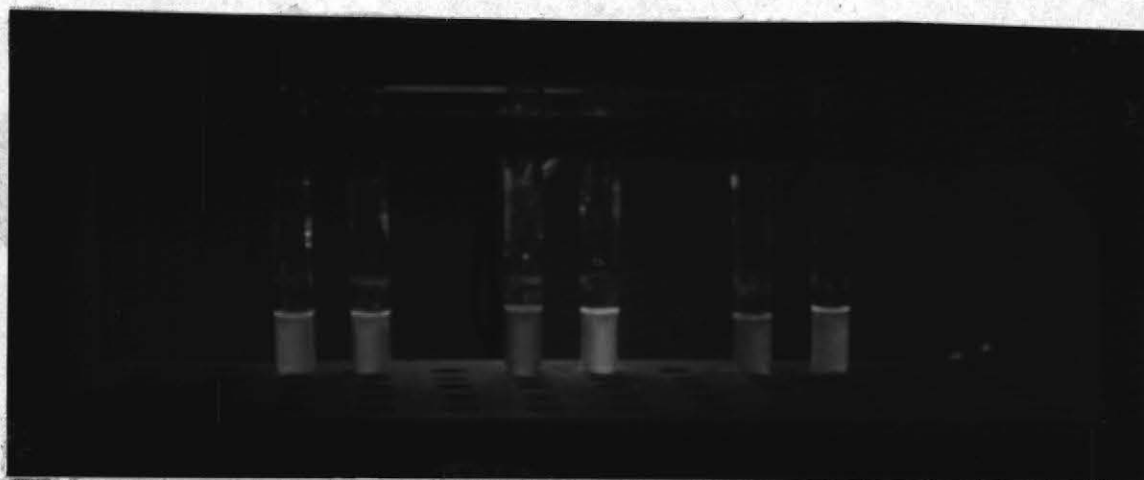


Figure 3. Fluorescent colors developed from sweetclover seeds. Left pair high coumarin, middle pair intermediate coumarin and right pair low coumarin. The right tube of each pair received 3 mg. of supplemental chlorophyll in the form of dried alfalfa leaves.

Printon print (14 seconds at 5 m.c.) from an Anseo Color transparency (F 4 at 7 minutes).

red colors. One mature alfalfa leaflet weighs approximately six to eight mg. and made a sufficient amount to add. This amount proved slightly better than the three mg. added in the tests previously conducted.

In testing the first eighty-five seeds, it was discovered that sufficient time should elapse in the dark room for the reader's eyes to adjust to the darkness. The trays of test tubes should also be exposed to the ultra violet light rays for at least five minutes so that all the coumarin can be thoroughly fluoresced. Too early readings led to the elimination of the first eighty-five seeds tested from the statistical analysis. Experience in reading the colors aids greatly in obtaining accurate results.

In the experiment 1030 seeds were tested; 710 lived and were checked by the plant tissue method for coumarin. Reducing the incidence of damping off could increase the ratio of livable plants obtained from seeds tested. The actual cutting of the seed made it imperative that the seed be germinated and planted within a relatively short time. Removing part of the cotyledons seemed to have little effect on germination or subsequent growth of the seedling.

This new seed test can measure the coumarin content of the seed with good accuracy. The seeds can then be germinated, planted and grown to maturity. Any seeds failing to germinate properly can be discarded.

The chi-square value obtained demonstrated a close relationship between the seed test and the plant tissue

test for coumarin. The high or low coumarin content prediction of the seed test gave results closely allied to those of the later plant tissue test. (Removal of the seed coat from the seed samples eliminated any possibility of maternal influence upon the results.) At present this new seed test represents a quick, accurate method of predicting coumarin content without destruction of the seed.

WESTERN BOND

FRAGILE CONTENT

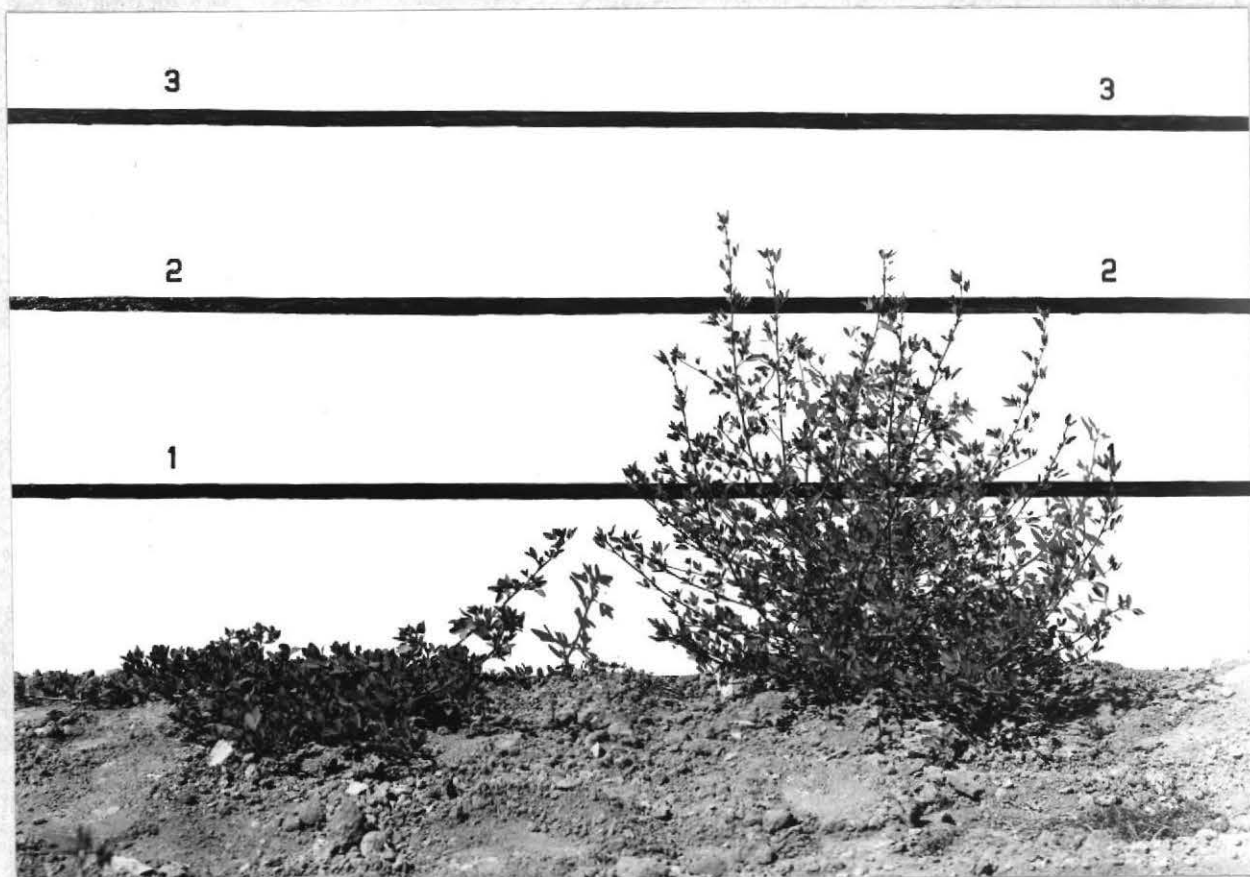


Figure 4. Dentata-like (left) and alba-like (right) F_3 progeny from the dentata alba F_1 X alba backcross. Evans Forage Farm, Utah Experiment Station, 1954.

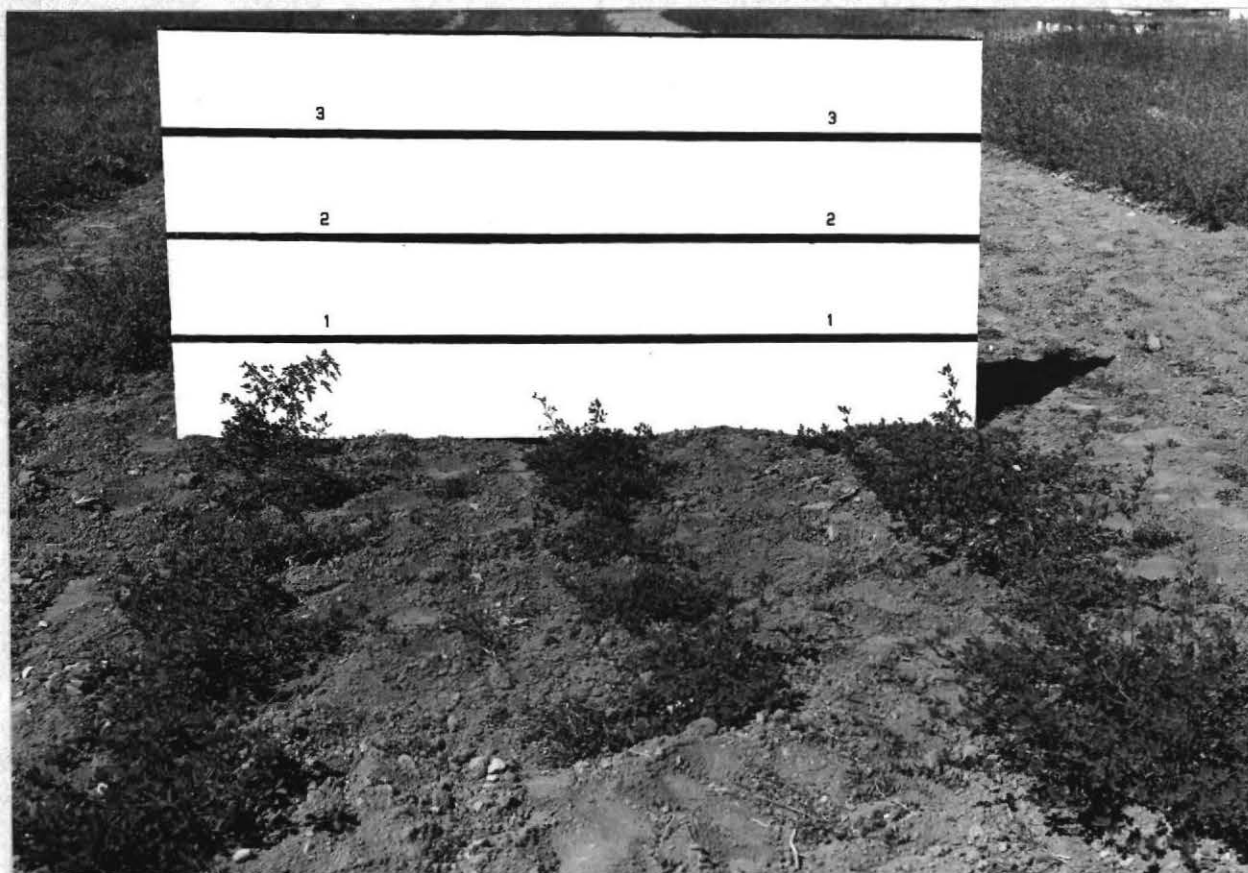


Figure 5. Dentata-like F_3 progeny from the dentata alba F_1 X alba backcross. Although low in coumarin content, these plants do not produce sufficient forage to be of agronomic value. Evans Forage Farm, Utah Experiment Station, 1954.

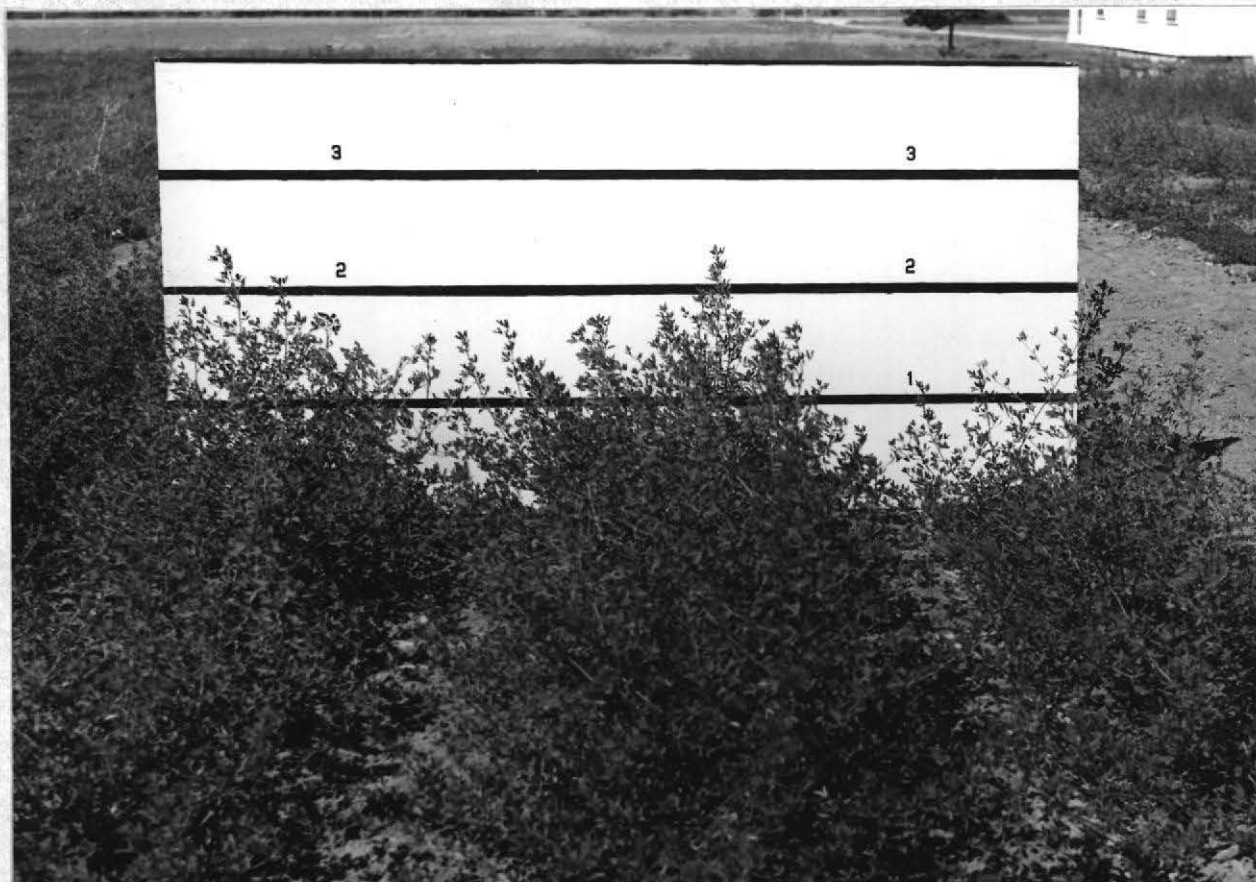


Figure 6. Alba-like F_3 progeny from the dentata alba F_1 X alba backcross. Yielding ability, leafiness, quick growth and low coumarin make these desirable agronomic types. Evans Forage Farm, Utah Experiment Station, 1954.

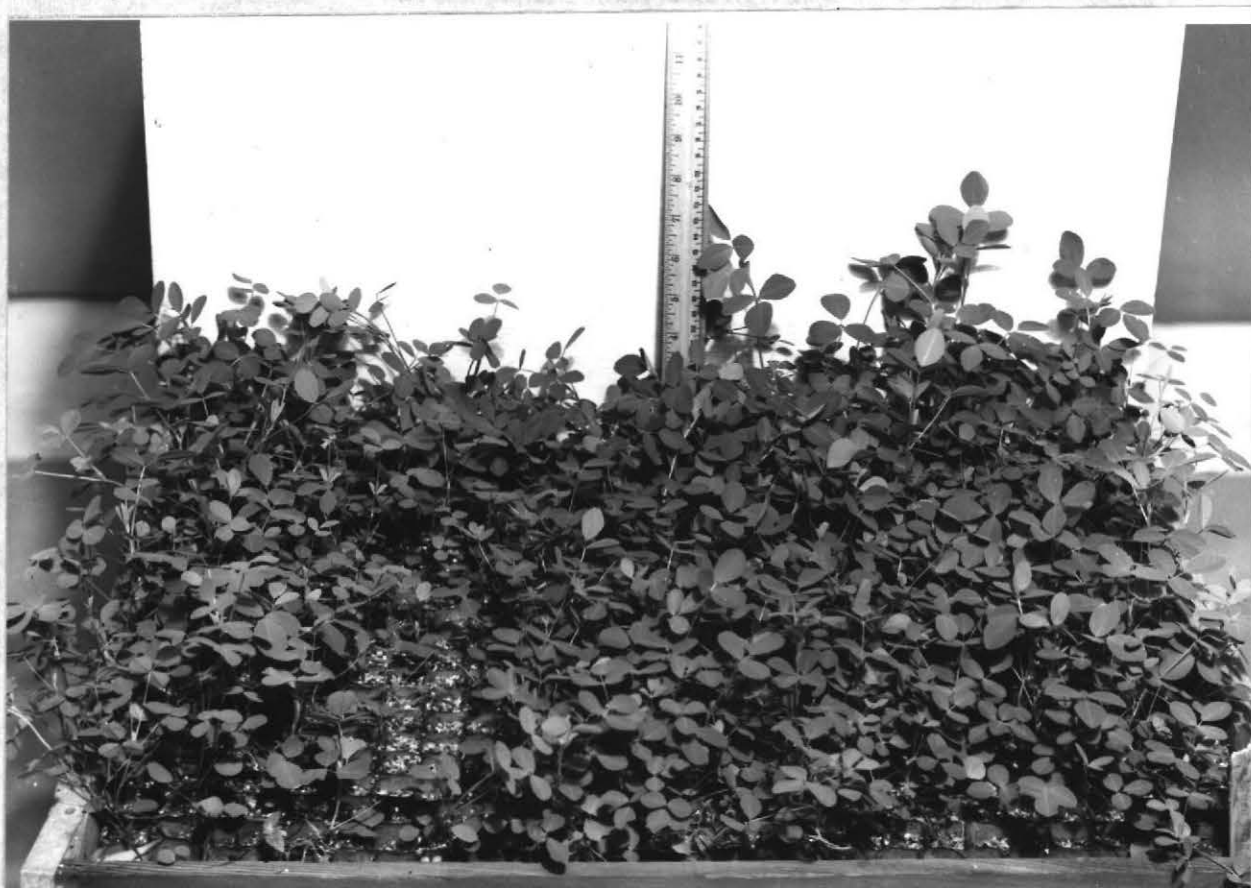


Figure 7. Greenhouse-grown sweetclover seedlings produced from coumarin tested seed. Seedlings of this size were sampled for plant tissue coumarin tests.

SUMMARY

1. Research was initiated to develop a technique for detecting coumarin in seeds of Melilotus spp. without destroying seed viability. Existing methods were studied, evaluated and any helpful points utilized in gaining this goal. The method devised was checked by running a plant tissue test for coumarin on the plants surviving seed sampling.

2. One thousand thirty seeds were dissected and samples tested for coumarin. Seeds were soaked in water at room temperature (26° C.) for 3 to 5 hours to start release of the bound coumarin in the seeds and to soften the seed coat for cutting. Each sample had the seed coat removed to eliminate possible maternal influences while being tested. Two ml. of 2.5 N NaOH plus three mg. of alfalfa leaf were added to the samples. The samples were then read and rated under a Burton ultra violet black light (shielded against visible light) in a dark room. The remaining portion of the seed was germinated, planted and grown to sufficient size for a sample of twenty mg. of leaf tissue to be removed for testing. Seven hundred ten plants grew and matured out of the 1030 seeds planted.

3. The results indicated that the seed tests and plant tissue tests for coumarin were highly correlated. Some of

the seed tests varied from the results of the plant tissue test, but sufficient error was not incurred to be serious. Reading the seed samples under ultra violet light requires more experience than reading plant tissue tests because of the greater variability of the color range and the necessity of waiting for the fluorescence to develop.

4. Dissecting of the seed and removal of part of the cotyledons did not reduce seedling vigor to any great extent. Most of the seedlings grew and developed normally with most of the losses due to damping off. Once the seeds were dissected it was necessary to plant them within a short time.

5. Results of the experimental data indicated that a practical seed test had been achieved. It is rapid, inexpensive, and coumarin content can be detected with a high degree of accuracy. This test can predict the inheritance of the seed from the sample taken and the remaining portion of the seeds desirable for crossing can be planted. In crosses involving plants of high and low coumarin, the seed test will be of little value; but in crossing or selfing the F_1 hybrids, a seed test would eliminate approximately three-fourths of the seeds produced on the coumarin factor alone. This makes it possible to save greenhouse space, labor in planting and may possibly speed up such a breeding program.

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