RUMEN IN VITRO STUDIES OF ALFALFA

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ABSTRACT

RUMEN *IN VITRO* STUDIES OF ALFALFA

by John S. Shenk, II

Four alfalfa populations were analyzed by a six hour *in vitro* technique.

The analysis of sixty-four in-place clones of the variety Tuna over a two year period indicated that real differences existed among clone means, although environmental influence and interaction does occur. Insect and disease resistance did not seem to be related to % DMD (per cent dry matter disappearance) in the MSA-C4 and MSB-C4 populations. The mean % DMD values for these populations were equal to or higher than the susceptible Tuna population. Correlations between yield and % DMD were nonsignificant in any of these three populations.

A population of selected alfalfa clones, moved from the field to the greenhouse over the winter and re-evaluated in the field the following year, were inconsistent in their DMD response. Plants placed in abnormal environmental situations may need a

period of adjustment before valid DMD comparisons can be made.

Chemical or biological treatment of forage is effective in increasing % DMD. NaOH pretreatment gave the greatest overall response; however, the increase was in solubility and not microbial digestibility.

Preliminary screening of large populations of clones with this in vitro technique can be effectively executed with only one determination per sample.

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By

John S. Shenk, II

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INTRODUCTION

The forage breeder, attempting to improve the nutritive value of forage, is beset with many obstacles. He must decide what characteristics contribute the most to nutritive value, devise or modify a selection technique until he is capable of measuring these systems accurately, and then select parental clones, stable in this character, but agronomically acceptable.

The lignin-cellulose complex is believed to be one of the plant systems responsible for nutritive value, but as yet its expression is not completely understood. Chemical or biological measurements of this character are possible; however, since chemical measurements will need to be interpreted in biological terms, in vitro systems have been suggested as a suitable alternative. Bioassays of this type have only recently been applied to plant selection.

The genetic system controlling the nutritive value of perennial forage plants is complex and subject to environmental influence; therefore, parental clones can best be selected by screening large populations of clones over a period of years. Only after relatively stable clones have been positively identified can the

plant breeder study this nutritive value character. Preliminary studies were conducted by Allinson (1966).

The objective of this research was to determine the relationship between DMD, environment, and agronomic characters in four different alfalfa plant populations. A preliminary attempt was also made to increase the % DMD of a forage by chemical or biological treatment. A pilot study was run on the number of sample determinations required for plant selection in large populations.

LITERATURE REVIEW

Improvement of Alfalfa Quality

Alfalfa quality is difficult to define. From a nutritional standpoint it is superior to many forages. The protein level is high and of relatively good quality. Methionine is the only amino acid that may be limiting. It has a high potential energy source, cellulose, but a rather low free sugar and starch content. Adequate minerals are present, especially calcium, and with the exception of B_{12}, it is considered a rich source of vitamins (Bolton, 1952).

Excellent references on the chemical composition and feeding value of alfalfa have been published by Morrison (1956), Maynard and Loosli (1956), and Crampton (1956); however, forage composition, as determined by standard chemical procedures, does not necessarily indicate biological activity.

Schillinger and Elliott (1966) isolated an antimetabolite substance from individual alfalfa plants. Although these plants were similar in chemical composition to plants without the antimetabolite, their toxicity was demonstrated in three biological assays.

Hanson et al. (1963), studying the saponin content of four alfalfa varieties (Buffalo, Ranger, Lahontan, and Vernal) from

eight locations in the United States, found differences among the varieties. Lahontan had the lowest saponin content and DuPuits the highest.

Substances have been extracted from alfalfa which are believed to affect vitamin utilization in growth. Pudelkiewicz and Matterson (1960) indicated that an inhibitor was present in the lipid portion of the separation. Sterol compounds did not appear to effect its reaction nor did the fibrous particles related to the lignin fraction. They postulated the action of the inhibitor was to prevent the utilization of tocopherol, a precurser of vitamin E.

Ayala et al. (1951) extracted an inhibitor from dried alfalfa juice that was not destroyed by heat or chemical treatments. They found that the inhibitor was present in greater concentrations in the dried juice than in the dehydrated meal; moreover, the addition of vitamin B_{12} was effective in partially overcoming the inhibitor effect.

No two alfalfa plants have the same genotype unless asexually propagated; therefore, the potential for diversity within varieties and species should be great. Elliott (1963) suggested that the unit of selection in alfalfa populations should be the individual clone. Individual plant selection was used by Bolton and Cormack (1953) to develop the varieties Saskatoon, and Viking from Grimm. The quantitative characters of winter hardiness and yield were

the criteria used to select these clones from the Grimm population.

An early study by Tisdale et al. (1950) indicated that inherent differences existed between the methionine content of two alfalfa clones. Singleton et al. (1952), evaluating one hundred clones for methionine content, could not establish variety differences, but found high and low clones within the population.

The idea of removing toxic substances with a breeding program is not new. Atwood and Sullivan (1943), using a combination of chemical identification and plant breeding techniques, removed the presence of HCN from a ladino clover strain. Allinson (1966) found that forage from a single alfalfa clone could cause a differential response when analyzed by two entirely different bioassay systems. Any breeding program using chemical or bioassay analysis as a selection tool will be limited by the sensitivity of the assay to measure the inhibitors within the forage; however, the ultimate goal of the breeding program will determine which type of assay or assays should be used.

Bioassay techniques presently employed range from small animals to specific strains of microorganisms. Elliott (1963) derived a means of selecting individual alfalfa clones with the meadow vole. Enzyme systems have been used to predict the nutritive value of forages by Donefer et al. (1963) In addition, enzymes are

presently being used to measure the biological activity of alfalfa leaf proteins (Gil et al., 1967).

Pederson et al. (1963), using Tricoderma and chicks as bioassays, investigated the activity of saponins. Oldfield et al. (1960) used dehydrated alfalfa samples containing the relative potencies of 119 mg. of coumestrol in lamb feeding trials. Artificial rumen systems have recently been applied by Schillinger (1965), Allinson (1966), and Gil et al. (1967), to evaluate individual alfalfa clones and their progenies.

Barnes et al. (1964), El-Shazly et al. (1960) and Barnes (1965) have published excellent reviews on the different in vitro techniques. Allinson (1966) used a six hour in vitro technique to screen large numbers of individual alfalfa clones. Clones of different nutritive value were differentially susceptible to lignin extraction. Lignin was significantly more extractable from clones of a high nutritive value.

Appraisal of In Vitro Techniques

Significant correlations have been found between in vitro and in vivo digestibility. Bowden and Church (1962b) analyzed forage samples for which live animal digestion coefficients were determined with sheep prior to in vitro digestion. Baumgardt et al. (1962), using forage evaluated with dairy heifers and sheep, correlated the

digestible organic matter, dry matter, and energy, to an in vitro system. Highest correlations were obtained with samples analyzed shortly after they were harvested and ground (Bowden and Church, 1962a).

Ingalls (1964) found significant correlations between the 6 hour in vitro technique and the nutritive value index; 36 hour fermentation was correlated with total animal digestion. Allinson (1966) concluded that the 6 hour fermentation technique was an effective tool for predicting the nutritive value of a forage.

Day to day variation was significantly less for a 48 hour fermentation than any other period tested (Baumgardt and Hi Kon Ho, 1964). Baumgardt et al. (1962) incorporated a standard forage in each fermentation trial to adjust for this variation.

El-Shazly et al. (1960) evaluated three different types of apparatus differing in the complexity and mode of action. A simple all glass system devised by Bentley et al. (1954), a semipermeable membrane system suggested by Warner (1956), and a continuous flow system modified from Warner's were tested. Only minor differences existed in the values obtained; therefore, the all glass system was preferred because of its simplicity. Simple, but efficient, techniques were advocated by Walker (1959). He stated that complexity was no criteria for obtaining agreement between digestibility values in in vitro and in vivo determinations.

The small quantity of forage, and the large number of samples that can be included in a single fermentation are examples of the practical application of this system; however, standardization of grinding within the laboratory is important (Baumgardt and Hi Kon Ho, 1964). The in vitro digestibility of grasses was increased to a greater extent by fine grinding (60 mesh vs. 40 mesh) than alfalfa digestibility (Baumgardt and Hi Kon Ho, 1964). One fineness of grind had no advantage over any other, nevertheless, error occurred if forage samples ground to pass through a 40 mesh sieve were compared to a 60 mesh.

The important role of solubility was pointed out by Donefer et al. (1963). As much as 29.3% of mature alfalfa forage was water soluble; therefore, solubility differences among forages may be erroneously interpreted as differences in digestibility. Microbial digestion may also be influenced by the rate at which carbohydrates become available to the fermentation media (Allinson, 1966).

Digestibility and the Lignin-Cellulose Complex

Forbes and Garrigus (1949), conducting a series of seven digestion trials with steers and wethers, found a straight line relationship between lignin content and dry matter digestibility. Highly significant negative correlations between lignin content and

digestibility were shown by Sullivan (1955). This relationship was found between digestion coefficients obtained from sheep feces and chemical lignin analysis of the same forage. Tomlin et al. (1965) concluded that lignin content was negatively correlated with in vitro cellulose digestibility at the 12 hour period for both grasses and legumes; moreover, the regression equations were significantly different.

As early as 1939 Crampton suggested that physical encrustation of the cellulose structure by lignin resulted in reduced digestibility. Dehority et al. (1962) suggested a lignin carbohydrate compound of plant cell wall material. It is known that lignin forms a dense membranous layer around the cell and even penetrates within the microfibrils, chemically combining with the crystalline cellulose. Bolkei (1963) reported the first physical evidence that acetal or hemiacetal bonds existed between the carbonyl groups of lignin and the hydroxyl groups of some portions of the holocellulose in wood and pulp lignin.

Van Soest (1962) demonstrated that alfalfa had a higher lignin content than grasses of equal digestibility; however, grass lignin is more easily dissolved. Allinson (1966), using ultraviolet spectrophotometry, found different absorption spectra for alfalfa clones of high and low nutritive value. The lignin polymer isolated from leaf and stem portions of timothy has different

functional groups (Stafford, 1962). Further studies by Stafford (1964) indicated that lignin in grasses was of two general types: One, an acid lignin polymer of ferulic acid and, two, a classical lignin polymer of coniferyl aldehyde or alcohol.

Pigden and Heinrichs (1957), studying the lignin content of six clonal lines of wheat grass, Agropyron intermedium, concluded that environmental factors affect lignin formation in plant tissue. Moisture was postulated as the primary environmental factor. Allinson (1966) found variability, both within and between genotypes, expressed as % DMD.

MATERIALS AND METHODS

The alfalfa clones used in this study were obtained from six populations. Clones with a different germplasm source and different types of management were grown from 1964 to 1966. Clones were harvested at the 1/2 bloom stage.

Plant Sources and Management

The unselected Tuna population consisted of 64 space-planted clones, randomly chosen from a larger population of 110 clones. These clones were grown in the field from 1964 through 1966. Only five were collected for testing from the first cutting, June 19, 1966. The second and third harvest of all 64 clones was made July 30 and September 14, 1966.

Seeds from two unrelated insect and disease resistant populations, MSA-C4 and MSB-C4, which had undergone fourteen cycles of recurrent phenotypic selection in eastern United States, were planted in the greenhouse April, 1966.[1] On June 19 all seedlings were flowering. Forage from each plant was harvested

[1] MSA-C4 and MSB-C4 germplasm pools were released in 1965 by the Crops and Research Division, U. S. Dept. of Agriculture, and the North Carolina Agricultural Experiment Station.

separately and then the plants were transplanted to the field. The first field harvest was made September 19, 1966.

Ten Vernal, five DuPuits and eight Culver clones constituted the selected population. High and low % DMD clones were selected by Allinson in 1965 from a large source nursery of varieties. The selected plants were moved into the greenhouse during the winter of 1965-66. All clones were replanted in June, 1966. Harvests were made July 19 and September 13, 1966.

Analysis Technique for Six Hour In Vitro DMD

Forage samples were analyzed by the six hour *in vitro* technique outlined by Allinson (1966). This procedure was based on the work of Bowden and Church (1962a), Baumgardt *et al.* (1962), and modifications by Ingalls (1964).

Individual alfalfa clones were harvested by hand, the forage dried at 44 C. for 48 hours, and ground with a Wiley mill (40 mesh screen). Samples were analyzed immediately or stored in plastic bags in a cold chamber at 4 to 5 C.

One g. duplicate samples from each clone were weighed into 125 ml. Erlenmeyer flasks to prepare for the fermentation. This was done one day before the actual test. The moisture content of the sample was taken by normal procedure.

To each flask was added 20 ml. of a phosphate-urea-carbonate buffer. The buffer contained 2,000 ml. water, 8.2 g. monobasic potassium phosphate, 17.4 g. dibasic sodium phosphate, 4.0 g. urea, and 7.4 g. monohydrate sodium carbonate. Adjustment was sometimes necessary with carbon dioxide to attain the 6.8-6.9 pH required. During this one hour time interval, rumen inoculum was collected from the cow in the following manner.

One hour after morning feeding time and one hour before collection the Holstein cow was denied free access to food and water. Rumen fluid was removed by hand and strained through three layers of cheesecloth into a preheated thermos jug.

The inoculum was placed in one liter vacuum flasks in the laboratory and maintained in a 39 C. water bath for 30 minutes. During the 30 minute interval, the fluid separated into two layers. The lower layer, containing the active portion of the mixture, was siphoned off and bubbled with carbon dioxide for three minutes.

Fermentation was initiated by adding 24 ml. of rumen inoculum to each buffered forage sample. Carbon dioxide was immediately added to the flask and the flask was sealed with a Bunsen valve stopper. Flask, sample, buffer, and inoculum were placed into a 39 C. water bath for the fermentation period. The addition of four drops of 20% thymol, six hours later, stopped all microbial action. Flasks were refrigerated until the following day.

Filtration was accomplished with predried and weighed crucibles fitted with frittered glass and a layer of Solka Floc. Crucibles were dried in a forced draft oven for 36 hours at 80 C. and then weighed to determine the dry weight of forage remaining. The % DMD was determined by correcting for inoculum error and moisture.

Forage Treatment and Antidote Procedure

1. Duplicate samples of forage T46 were treated four different ways 18 hours before fermentation. The treatments were applied to 1 g. forage samples as follows: (a) 6 ml. of 0.5 N NaOH for 18 hours then neutralized before fermentation with HCL, (b) 6 ml. of 0.5 N NaOH neutralized with HCL and 25 mg. of trypsin and 19 mg. pepsin added before fermentation, (c) 5 ml. of water plus 0.1 ml. cellulase. The untreated (none) sample was analyzed by the standard DMD procedure.

2. Forage T46 pretreated 12 hours. Solutions were removed and antidotes added one hour before fermentation. This test consisted of treating 10 g. of T46 forage 12 hours before fermentation with (1) 100 ml. 0.5 N NaOH, (2) 60 mg. each of trypsin and pepsin plus 100 ml. of water, (3) 0.15 ml. of cellulase and 100 ml. of water, (4) 100 ml. water. At the end of the pretreatment period each sample was filtered and washed with distilled

water, dried at 60 C., reweighed in 1 g. duplicate samples 24 hours later, and antidoted. The antidotes consisted of 50 mg. hydrostate casein, 50 mg. of vitamin trace mineral mix, and 50 mg. of urea. Each of these substances was added immediately before the inoculum fluid.

3. Response of three Tuna forages differing in % DMD to two different NaOH pretreatments. The experiment consisted of two parts carried out simultaneously.

All duplicate samples in the first group were pretreated for their specified time period and then inoculated with rumen fluid. (1) 0.5 N NaOH was added to forage samples of clones T95 and T108 and allowed to stand for 12 hours at room temperature before neutralizing with HCL. Forage T46 was pretreated similarly for 18 hours. (2) These samples were pretreated the same as (1) except the neutralized samples were dried at 60 C. for 24 hours before fermentation. (3) No pretreatment employed, standard DMD procedure.

Duplicate samples in the second group were pretreated in the same manner as the first group; however, no inoculum was added. This method was used to establish a correction factor to derive the actual % digested by the rumen fluid.

Statistical Methods

The fermentation variation from day to day and week to week was estimated in the DMD technique by including a control

sample in each fermentation. The mean value for these control samples was used to adjust the % DMD of each forage sample (Spragg, 1920).

A partially nested analysis of variance was used to estimate the standard error of the mean of four, two, and one determinations per sample.

Forage from five clones of three varieties was used for this analysis. On July 21, 1966, duplicate samples of the forage from these 15 clones were analyzed by the DMD procedure. Likewise, August 4, 1966, the same forage samples were re-evaluated in a similar manner, giving a total of four determination values for each of the 15 clones.

The standard error of the mean for four determinations was calculated, and estimates of the standard error of the mean of two and one determinations were obtained by changing the denomination.

The proportional analysis of variance used on the Tuna population was explained by Snedecor (1956). This technique was employed because data was only available from one cutting in 1965 and two cuttings in 1966. The interaction term, years x clone (y) was used to estimate the environmental error.

RESULTS

Tuna Population

Table 1 contains the means and ranges for the % DMD of each cutting and year. The % DMD range decreased from 1965 to 1966 and decreased further from July to September, 1966. A major % DMD increase occurred in the clone population between 1965 and 1966. This increase was evident in the June, 1966, harvest also, but only five clones of the total population were sampled. Another smaller increase in % DMD was observed between July and September, 1966. The histograms, Figure 1, indicate the normality of the population and the decrease in % DMD range, and the increase in % DMD population mean over the three harvests.

Table 1. -- 1965-66 cutting means and ranges of the % DMD values for 64 unselected Tuna clones.

Variety	No.[a]	Harvest Date					
		June, 1965[b]		July 30, 1966		Sept. 14, 1966	
		Range	Mean	Range	Mean	Range	Mean
Tuna	64	22.8-40.6	32.3	33.3-50.5	40.6	37.7-49.2	42.7

[a]Number of clones in study.

[b]% DMD values by Allinson (1966).

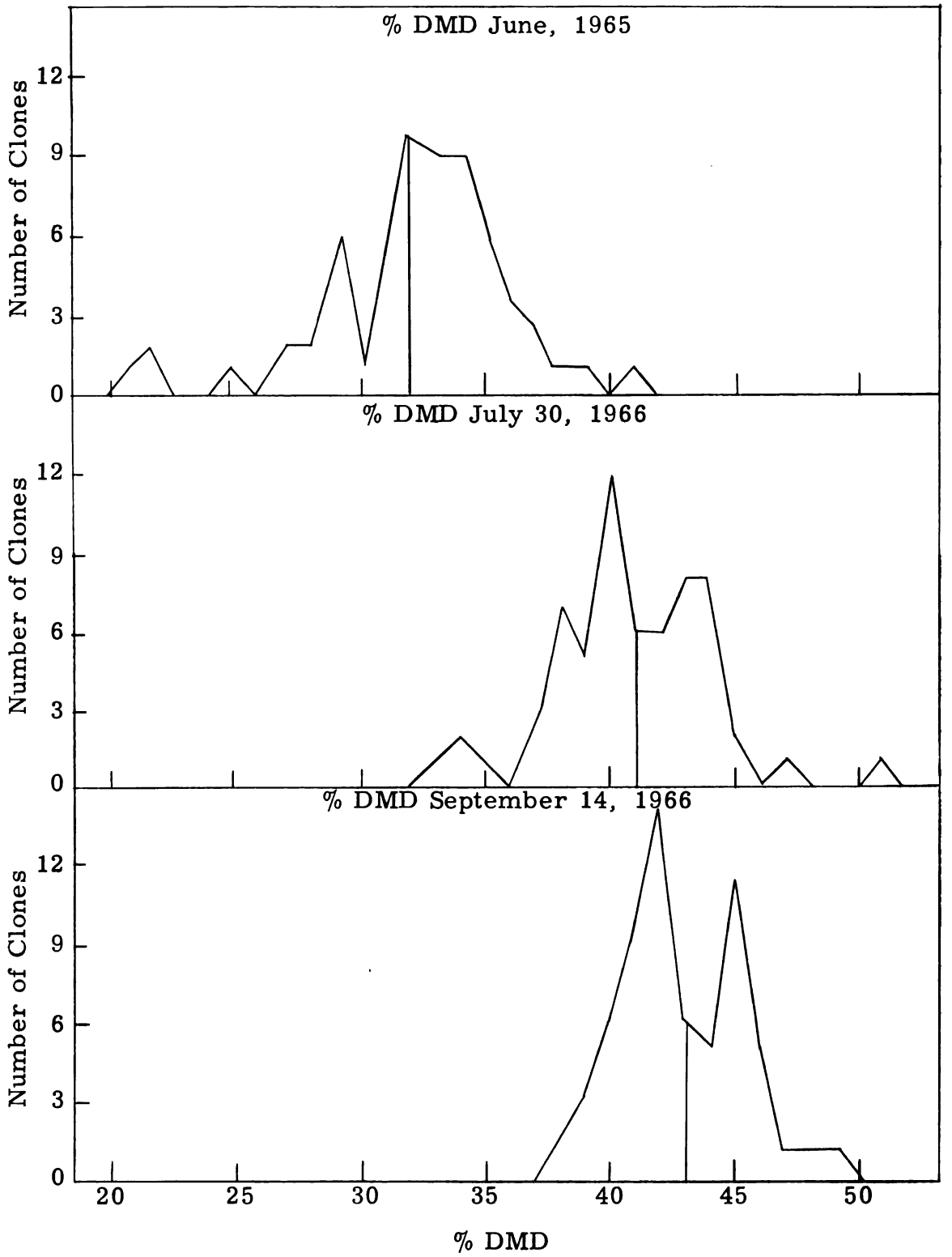

Figure 1. -- Histograms of the unselected Tuna clones for three harvests and two years. % DMD values used to measure the population.

Proportional analysis of variance was applied to this population. The average response for the two years was quite different although no appropriate error term for making a test exists in this data. The relevant question is whether the clones tend to be ranked the same from year to year. Table 2 shows that the sums of squares for the year variance were very large. The F statistic calculated for the clone effects was significant at 1%, indicating real differences existed among the clonal means. The year x clone (y) interaction was significant at 6%. Variation in the % DMD of a given clone was influenced to some extent by factors causing this interaction. For example, of the four clones in the top 5% of the % DMD distribution in 1965, two were still in this group at the end of 1966; moreover, of the four clones in the bottom 5% of the distribution, one clone was still in this group at the end of 1966.

Table 2. -- Proportional analysis of variance of the % DMD values in an unselected Tuna population.

Source of Variance	df	SS	MS	F
Total	191	5,749.61		
Year	1	3,753.74		
Cuttings (y)	1	141.96		
Clone	63	952.08	15.11	2.66[a]
Year x clone (y)	63	544.41	8.64	1.52[b]
Error	63	357.42	5.67	

[a]Significant at 1% level.

[b]Significant at 6% level.

Correlations between the % DMD for each of the Tuna clones, forage dry weight, and clone color are represented in Table 3. Clonal dry weight was not significantly correlated with % DMD. Significant positive correlations were obtained between the clonal dry weight of the July and September cuttings, and clone color and dry weight of the September cutting.

Table 3. -- Correlation values between characteristics of the Tuna population in 1965-66.

Correlations	r Value
June, 1965, Harvest	
% DMD vs. Dry Wt.[a]	0.0211
July 30, 1966, Harvest	
% DMD vs. Dry Wt.	0.0971
September 14, 1966, Harvest	
% DMD vs. Dry Wt.[c]	0.1129
% DMD vs. Color[c, d]	0.2201
Dry Wt. vs. Color[c, d]	0.7569[e]
July 30 Harvest and September 14 Harvest	
Dry Wt. vs. Dry Wt.[b]	0.6195[e]

[a] 110 Clone comparisons (Allinson, 1966).

[b] 64 Clone comparisons.

[c] 20 Clone comparisons.

[d] Color rating 10 green, 1 yellow

[e] Significant at 1% level.

MSA-C4 and MSB-C4 Populations

These two populations were chosen for evaluation because of their insect and disease resistant qualities and their exceptional dark green foliage. Table 4 contains the means and ranges for the % DMD of the plants for both harvests, as well as simple correlations between % DMD, dry weight, and color.

Table 4. -- Means and ranges for the % DMD of MSA-C4 and MSB-C4 populations. Simple correlations between % DMD, dry weight, color, and harvests.

Population	Number of Clones	Mean	Range
June Greenhouse Harvest			
MSA-C4	40	35.9	30.0-44.2
MSB-C4	54	36.6	28.1-46.3
September Field Harvest			
MSA-C4	46	41.3	35.5-47.9
MSB-C4	70	45.3	38.7-51.6

Population	Correlations	r Value
MSA-C4	% DMD and Forage Weight	-0.2278
	% DMD and Forage Color[a]	-0.2545
	% DMD Greenhouse and Field	0.1924
MSB-C4	% DMD and Forage Weight	0.0075
	% DMD and Forage Color[a]	0.1669
	% DMD Greenhouse and Field	0.1078

[a]Color rating--10 green, 1 yellow.

The populations were near normal in their distributions, Figure 2, but differed in the range and mean of their % DMD from greenhouse to field; nevertheless, the % DMD values for the September field cutting of both populations were similar to those of the Tuna population harvested in September. Even though the greenhouse plants were only twelve to sixteen inches high when harvested, with a total dry weight of a few grams, their mean % DMD was 8.7-5.4% lower.

All correlations were non-significant, but the correlation values calculated between % DMD, dry weight, and foliage color differed in sign from population to population. The insignificance of their value obtained between the % DMD of the greenhouse seedlings and the field clones needs further verification in 1967.

Vernal, DuPuits, and Culver Clones

This population consisted of 10 Vernal, 8 Culver, and 5 DuPuits clones. Table 5 contains the range and the mean % DMD values for the selected high and low clones of each variety. Changes in the mean values and ranges from year to year and cutting to cutting are evident; however, the clones in the low selected group made the greatest change from 1965 to 1966. This is illustrated graphically in Figure 3. The % DMD values of all clones in 1966 were grouped together in the high range; in addition, the range

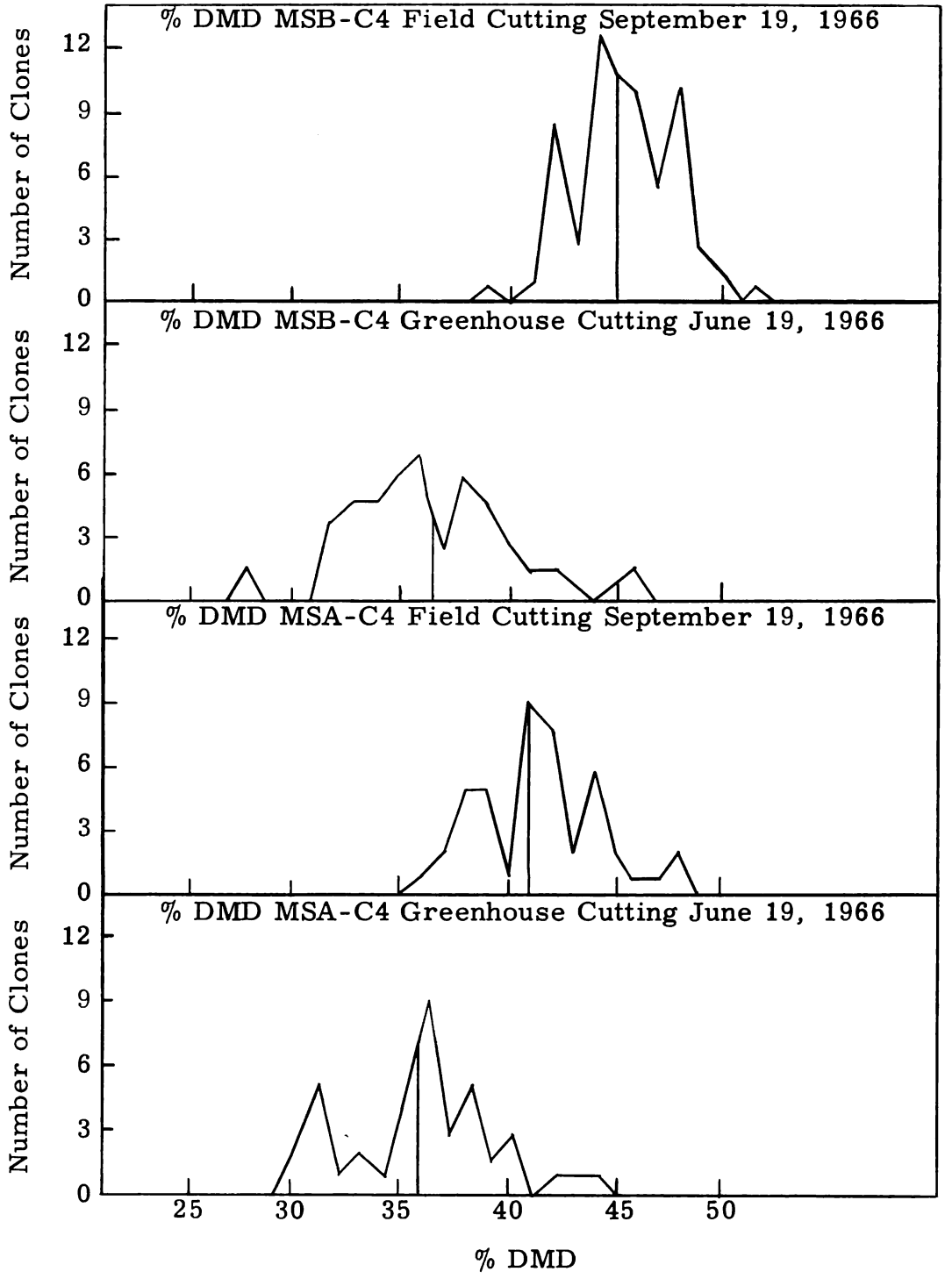

Figure 2. -- Histograms of the MSA-C4 and MSB-C4 field and greenhouse populations. % DMD values used to measure the population.

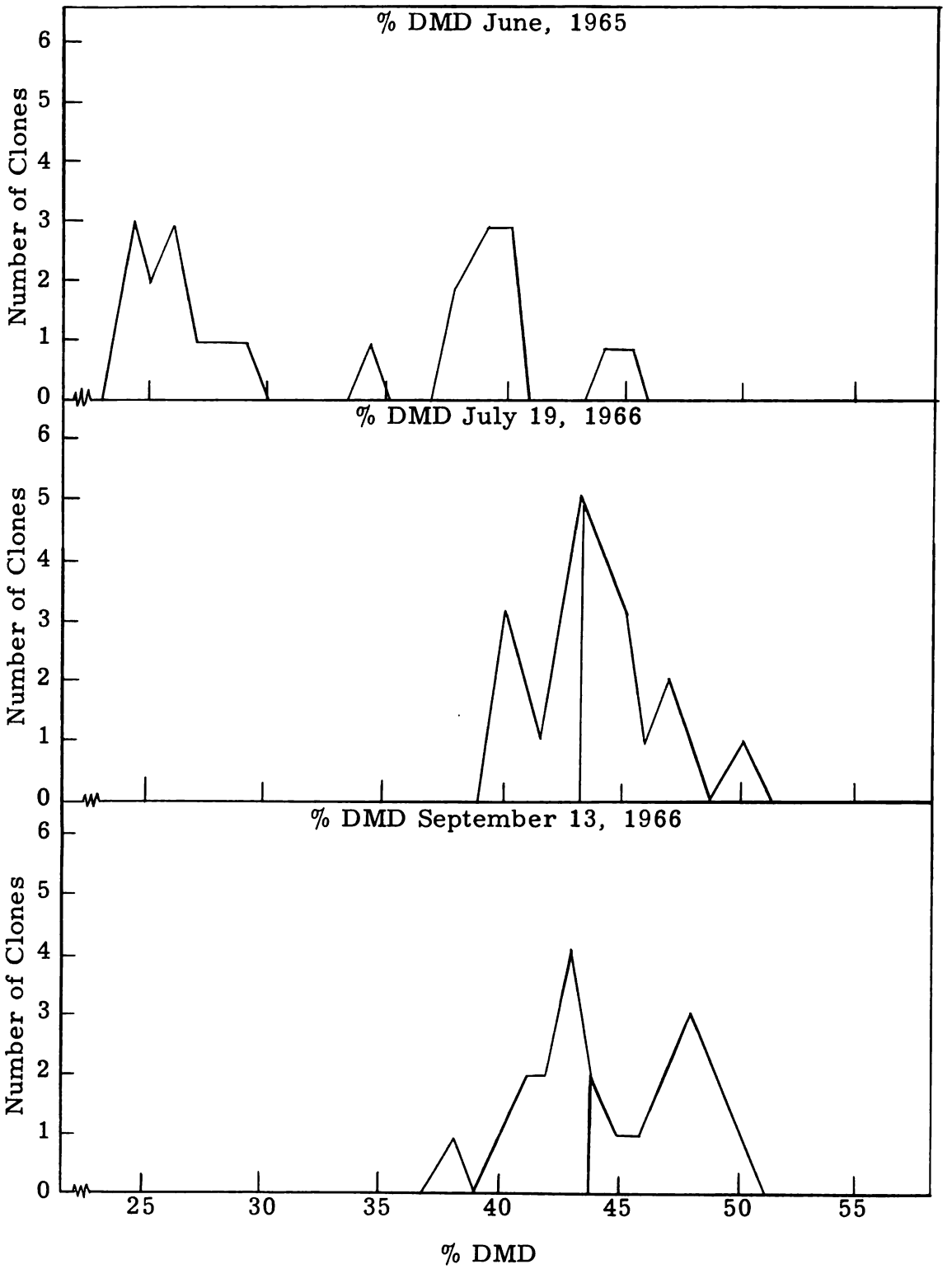

Figure 3. -- Histograms of the selected clones for three harvests and two years. % DMD values used to measure the population.

among the clones decreases. Clonal changes between cuttings in 1966 were rather small in comparison to the year to year changes.

Table 5. -- Range, mean, and number of clones in each variety represented in the selected population. Varieties divided further into sub-groups high and low. Values expressed as % DMD.

Variety No.[a]	Harvest Date					
	June, 1965[b]		July 19, 1966		Sept. 13, 1966	
	Range	Mean	Range	Mean	Range	Mean
Vernal						
High 6	34.3-40.0	38.4	43.5-50.4	46.5	41.3-49.6	46.1
Low 4	23.4-26.0	25.3	43.3-46.5	44.5	38.1-47.4	42.0
DuPuits						
High 2	44.2-45.0	44.6	40.2-42.4	41.3	44.7-42.6	43.7
Low	27.0-29.2	27.9	40.0-40.8	40.3	40.6-43.5	41.9
Culver						
High 3	37.9-40.2	38.8	41.2-44.4	42.9	44.1-45.8	43.8
Low 5	23.6-25.2	24.4	42.5-44.6	43.4	42.6-48.7	46.2
Population	23.4-45.0	33.2	40.0-50.4	43.2	38.1-49.6	44.0

[a]Number of clones in each sub-group.

[b]% DMD values by Allinson (1966).

Forage Treatment and DMD

Forage from alfalfa clone T46 was treated with two enzyme preparations: (1) cellulase or (2) a combination of trypsin

and pepsin. The % DMD was increased 3.1% and 4.8% respectively, Table 6. NaOH alone gave an 8.0% increase. Another slight increase was attained by adding trypsin and pepsin immediately before fermentation.

Table 6. -- % DMD for alfalfa forage T46 pretreated 18 hours.

Pretreatment[a]	% DMD	% Increase in DMD
NaOH 0.5N	47.4	8.0
NaOH 0.5N Trypsin, Pepsin	48.1	8.7
Cellulase	42.5	3.1
Trypsin, Pepsin	44.2	4.8
None[b]	39.4	---

[a]Concentrations and procedures listed in materials and methods.

[b]No pretreatment.

To determine the relative digestibility of the insoluble portion of forage T46 it was again pretreated 12 hours with .5N NaOH, cellulase, trypsin, pepsin, and water. All soluble substances were removed by filtration and the remaining portion was dried before rumen fermentation. Table 7 contains the results.

The organic and inorganic nitrogen sources gave near equal increases to the residue of each respective pretreatment.

The vitamin, trace mineral mix lowered the % DMD in every case except the NaOH pretreated sample; in addition, the NaOH pretreated sample had the lowest % DMD without the antidotes, 10.5%. When urea was added the % DMD increased to 19.6%. This is higher than any of the water soluble residue values with or without antidotes. The insoluble portion of the trypsin-pepsin pretreatment had the highest % DMD values, with only minor antidote effects.

Table 7.-- Forage T46 pretreated 12 hours. Solution removed and antidotes added one hour before fermentation. Values expressed as % DMD.

12 Hour Pretreatment Solution Removed					
Antidote	(0.5N NaOH)	Trypsin Pepsin	Cellulase	H_2O	Average
Hydrostat Casein	18.0	27.6	21.1	18.4	21.3
Vitamin Trace Min.	11.4	23.3	15.8	15.0	16.4
Urea	19.6	26.6	21.7	18.3	21.3
None[a]	10.5	26.8	20.2	17.9	18.9
Average	14.9	26.3	19.7	17.4	----

[a]No antidote added.

The NaOH pretreatment was selected for further study on three forage samples with different untreated % DMD values.

The untreated % DMD values, the % soluble in water, and the actual % digested by the rumen microorganisms are found in Table 8. These three forages not only differed in their % DMD but in the other values. Forage T95 had the highest water soluble portion and T46 the lowest, but T108, the intermediate clone, had the highest percent of its insoluble portion digested by the rumen inoculum.

Table 8. -- The % DMD, % water soluble, and % microbial digestion of three Tuna forages: T95, T108, and T46.

Clone	% DMD	% Soluble	% Digested[a]
T95	49.2	34.6	14.6
T108	45.7	28.3	17.4
T46	39.4	27.0	12.4

[a]% DMD minus % water soluble.

The effect of NaOH pretreatment on these three Tuna forages is found in the first half of Table 9. The second portion of the table was calculated by subtracting the untreated values for each forage (Table 8) from the treated (Table 9). All samples increased in % DMD and % soluble when treated with NaOH. The calculated % DMD increased 6.5 to 8.0 as the treated forage % DMD decreased 55.7 to 47.4; moreover, as the calculated solubility

values increased 6.2 to 14.9, the calculated percent digested by the rumen inoculum decreased 0.3 to -6.9. Forage T46 had the lowest treated % DMD, the greatest increase in calculated solubility, and the greatest decrease in calculated digestibility. Clone T95 was exactly the opposite and T108 was intermediate.

Table 9. -- The % DMD, % water soluble, and % microbial digestion of three Tuna forages pretreated with NaOH. Calculated values derived by subtracting the untreated values (Table 8) from the treated values.

	Treated Values			Calculated Values Treated Minus Untreated		
Clone	% DMD	% Soluble	% Digested[a]	% DMD[b]	% Soluble[c]	% Digested[d]
T95	55.7	40.8	14.9	6.5	6.2	0.3
T108	53.2	36.9	16.3	7.5	8.6	-1.1
T46	47.4	41.9	5.5	8.0	14.9	-6.9

[a]Treated % DMD minus treated % soluble.

[b]Treated % DMD minus untreated % DMD.

[c]Treated % soluble minus untreated % soluble.

[d]Remainder of b minus remainder of c.

A somewhat different trend occurred when the sample treated with NaOH was dried and then exposed to fermentation, Table 10. Another increase in % DMD occurred for each sample with a corresponding increase in solubility; however, both the

treated in % digested and the calculated were lower than in the undried NaOH treatments.

Table 10. -- The % DMD, % water soluble, and % microbial digestion of three Tuna forages pretreated with NaOH and dried before fermentation. Calculated values derived by subtracting the untreated (Table 8) from the treated values.

	Treated Values			Calculated Values Treated Minus Untreated		
Clone	% DMD	% Soluble	% Digested[a]	% DMD[b]	% Soluble[c]	% Digested[d]
T95	62.4	55.3	7.1	13.2	20.7	-7.5
T108	58.5	48.4	10.1	12.8	20.1	-7.3
T46	49.3	----	----	9.9	----	----

[a]Treated % DMD minus treated % soluble.

[b]Treated % DMD minus untreated % DMD.

[c]Treated % soluble minus untreated % soluble.

[d]Remainder of b minus remainder of c.

DMD Pilot Study

To determine the relative importance of clone and determination error variance, a partially nested analysis of variance was employed, Table 11. The variety main effect was not significant. The mean square value for the interaction term (block x clone [v]) was very small in comparison to the clone

mean square; moreover, it was noticeably smaller than the determination mean square. Legitimate pooling of the sums of squares for the interaction term and the determination was therefore possible, Formula (1).

Table 11. -- Partially nested analysis of variance table of DMD sampling pilot study.

Source of Variance	df	SS	MS	F
Total	59	289.010		
Block	1	3.504		
Variety	2	85.317	42.569	4.022
Block x Variety	2	21.212	10.606	
Clone (v)	12	155.051	12.921	22.668[a]
Time x Clone (v)	12	2.291	0.570 (1)	
Determinations	30	21.635		

[a]Significant at 1%.

(1) $$S_p^2 = \frac{SS_1 + SS_2}{df_1 + df_2} = 0.570$$

(2) $$S_{\bar{x}} = \sqrt{\frac{0.570}{4}} = 0.378$$

(3) $$S_{\bar{x}} = \sqrt{\frac{0.570}{2}} = 0.534$$

(4) $$S_{\bar{x}} = \sqrt{\frac{0.570}{1}} = 0.755$$

The calculated pooled variance was 0.570 with 42 degrees of freedom. The standard error of the clone pooled mean for four determinations per clone was calculated to be 0.378, Formula (2). The estimated standard error of the mean of two determinations was 0.534 and one determination 0.755, Formula 3 and 4. Applying these calculated values to Tukey's multiple range test, any two means differing by more than 1.90% would be judged significant at the 5% level with four determinations, in contrast to 3.77% if only one determination was made.

DISCUSSION

The four populations of alfalfa clones analyzed by the in vitro DMD technique approached normal distribution. Curves of this type are representative of quantitative genetic characters, with continuous variation. Since this bioassay technique is responsive to the solubility and digestibility of the lignin-cellulose complex (Halliwell and Bryant, 1963), the physiological development of this structural arrangement is possibly a multi-gene reaction. Hopefully, some degree of genetic control over this system may be possible which will permit an improvement in the overall nutritional value of alfalfa forage.

In order to make genetic advance by selection, two population parameters must be known or estimated: (1) The amount of genetic variability in a population and (2) the masking effect of environment or the interaction components of variation.

Agronomic factors which may be directly or indirectly affected by selection and development of plants with in vitro technique should also be considered. Alfalfa varieties with high quality combined with low forage yield would be of little use agriculturally;

likewise, low forage quality together with high insect and disease resistance would certainly not be agronomically acceptable.

Unselected Tuna Population

The DMD analysis of the Tuna population demonstrated three things: (1) Some environmental interaction does occur and must be considered; (2) Alfalfa clones should be analyzed over a period of at least two years with more than one cutting per year; (3) Yield and % DMD are not necessarily related.

The environmental influence was clearly shown by the increase in all % DMD values from 1965-1966. This effect is probably a combination of developments. A number of hypotheses can be offered to explain the reaction. Even though the same Holstein cow provided the rumen inoculum for all forage samples during both years of study, some physiological or microbial change within the cow could have occurred between the years. This view is supported to some degree in the literature, Baumgardt and Hi Kon Ho (1964).

The second hypothesis, changes in the physiological development of the plant, is more likely. Pigden and Heinrichs (1957), working with lignin content in wheatgrass, concluded that environmental changes could alter the formation of the lignin polymer. Allinson (1966) demonstrated that the % DMD can even vary within plants of the same genotype; therefore, with the potential buffering

capacity of the heterozygous genotype of alfalfa plants, it is entirely possible that the response of a clone to DMD analysis could vary considerably from one environmental situation to another.

Pigden and Heinrichs (1957) concluded further that moisture was the primary factor responsible for the environmental influence. Differences did exist in amount and distribution of rainfall from 1965 to 1966. Low moisture and dry conditions existed in 1965, with corresponding low % DMD values, and in 1966, with more normal moisture, clones had higher % DMD values.

Assuming the difference measured in % DMD for a clone over the two years and three cuttings was due primarily to environmental influence, the magnitude of its importance was estimated by the significance of the year x clone (y) interaction component, Table 2. The F. Value of the 1.53 was within .01 units of being significant at the 5% level, whereas the main effect of clone differences was significant at 1% level. The lack of a strong year x clone interaction leads to the conclusion that the clone means are a good indication of the genetic population for % DMD.

Selection of quantitative characteristics in perennial crops is best conducted over a period of years and a number of cuttings within a year. The additional information of years and cuttings is needed to more accurately estimate the plant's inherent stability under different environmental conditions.

The agronomic character of yield was compared to the % DMD values for the two cuttings in 1966. Allinson (1966) found a significant negative correlation between % DMD and yield of some varieties. The correlations in this study were low and approached zero; therefore, the relationship of % DMD to yield was small or nonexistent.

Disease and Insect Resistant MSA-C4 and MSB-C4 Population

The possibility of combining insect and disease resistance with high nutritional value was the primary reason for evaluating the MSA-C4 and MSB-C4 populations. The first analysis of these clones indicates that they are equal and perhaps superior to present varieties. The mean % DMD for the MSA-C4 clones was higher than any other group of clones tested. Any relationship between yield and % DMD could not be demonstrated in either population.

The attempt to predict field % DMD from greenhouse % DMD data shows little promise within the same year. Due to the low values achieved for these young greenhouse seedlings and the apparent distortion of the % DMD response of the twenty-three selected clones, DMD evaluation of alfalfa plants under greenhouse conditions must be viewed with caution. Further study is needed to gain a better understanding of the phenomena.

Selected Clone Population

The twenty-three clones representing three varieties in the selected population were separated into two distinct groups in 1965, Figure 3. After placing these clones under an abnormal winter environment in the greenhouse (18 hour photoperiod and 70-80 F. temperature), the population distribution curves for the July and September, 1966, field harvest again approached normality.

Such results may occur because of the alteration of the physiological processes within the plant distorting any re-evaluation or comparison the following year. As many as 63% of the clones could have been falsely classified, since their selection was on a single harvest basis.

The Solubility and Digestibility of Treated Forage

Chemical and biological treatment of forage has not been used to increase its efficiency by ruminants. If accomplished, it would have the advantage of immediate application, providing beneficial animal response could be economically demonstrated. Additional benefits from this approach would be knowledge about the factors in forage which limit or stimulate animal usage.

Forages pretreated with enzymes would be expected to increase forage breakdown, thereby increasing its % DMD.

Likewise NaOH, which has had extensive use as a lignin solvent (Stafford, 1962), should increase the % DMD of forage. These facts were verified in the initial experiment, Table 6. Values similar to these were reported by Donefer et al. (1963). Antidoting the insoluble portion of the forage with organic and inorganic nitrogen increased the digestibility in every case. The most impressive increase occurred when urea was added to the insoluble portion of the NaOH pretreatment, Table 7.

Forage solubility and microbial digestibility are combined in the % DMD values. Emphasis in most DMD studies is placed on microbial digestibility; however, the importance of the solubility factor can be seen in Table 8. Forage from clone T95 had the highest % DMD value but not the highest microbial digestion. Treatment with NaOH (Table 9) caused increases in all % DMD values in proportion to the untreated values; however, this increase was again due to solubility since the percent digested actually decreased. The greatest digestion reduction was exhibited by the forage with the lowest untreated % DMD value.

Evaporation of the water from the NaOH treated sample to resemble a dried forage caused further changes in solubility and digestion. The solubility again increased in all samples with a corresponding decrease in microbial digestion.

Increasing forage solubility may be important in overall animal nutrition even though rumen digestion by the microorganism may be reduced. If the increased availability of these nutrient substances would increase their assimilation into the animal's digestive system, pretreatment to increase solubility could be beneficial.

Suggested Changes in the DMD Mass Selection Technique

The application of the six hour DMD technique to mass selection of forage may be a valuable innovation. However, with large numbers of samples requiring analysis, any reduction in the sample number would be a worthwhile improvement.

Allinson (1966) suggested that since the variance between fermentations was greater than the variance within fermentation, samples should be evaluated in as many fermentations as possible. Duplication would be eliminated within fermentation. This would increase the error variance accuracy for a clone, but it would not reduce the number of samples.

The standard error of a clone mean using two determinations was 0.534. This value is comparable to those reported by Barnes (1965), and Allinson (1966). With the relatively small standard deviation for a single determination, 0.755, and the range in % DMD values reported in the preceding studies, identification of the upper 5% or 10% of the clones could be adequately

accomplished by one determination. The number of clones evaluated in a mass selection study is of much greater importance than the slight misclassification of a few. This would be especially valuable in analyzing perennials with multiple harvests.

SUMMARY AND CONCLUSIONS

Four alfalfa populations have been evaluated by the six hour in vitro technique. The unselected Tuna population yielded information about the environmental influence upon in-place field clones. The MSA-C4 and MSB-C4 populations indicated the possibilities of selecting superior clones within insect and disease limitations, and the selected population demonstrated the effects of abnormal environment on DMD re-evaluation.

Studies were conducted on the solubility and digestibility of chemically or biologically treated forage. Finally, a change was suggested in the application of the six hour in vitro procedure to a preliminary forage screening technique. The following conclusions may be stated.

1. Environmental influence and interaction does alter the DMD values of a space planted alfalfa population; however, selection of superior clones should be possible if the population is analyzed for more than one year and one cutting per year.
2. The agronomic characters of yield and disease and insect resistance are not necessarily related to DMD

in all populations. The genetic system responsible for these characters appears to be independent.

3. Alfalfa clones moved from the field to the greenhouse over the winter and re-evaluated in the field the following year were inconsistent in their DMD response. Abnormal environmental stress placed upon the plant for a prolonged period of time may require a period of adjustment before valid DMD comparisons can be made.
4. Forage solubility can be increased by chemical or biological treatment; however, its importance in animal nutrition has not been established.
5. Preliminary screening of large numbers of individual plants can be effectively carried out with the DMD technique by making only one determination per sample. This allows more clones, cuttings, and years to be evaluated with the same resources.

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