SELECTION AND CHARACTERIZATION OF DIGESTIBILITY DIFFERENCES IN ALFALFA BY CHEMICAL AND BIOASSAY PROCEDURES

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ABSTRACT

SELECTION AND CHARACTERIZATION OF DIGESTIBILITY DIFFERENCES IN ALFALFA BY CHEMICAL AND BIOASSAY PROCEDURES

By

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Clones with high and low six hour *in vitro* dry matter disappearance values (6H-DMD) were selected from and crossed within Vernal and DuPuits varieties. The progeny from clones with high 6H-DMD values had consistently higher 6H-DMD values than progeny from clones with low 6H-DMD values. Clones with stable high or low 6H-DMD values over two cuttings were identified in five source populations.

The correlations between clone yield and 6H-DMD values were negative or near zero. Cell-wall analysis of clones selected for a second cycle of improvement indicated a very close relationship between total cell-wall constituents (NDF) and 6H-DMD values of the forage.

Second cycle clones had significant general and specific combining abilities for yield as well as leaf and NDF content. Progeny from clones with high 6H-DMD

values were higher in yield and leaf content, but lower in NDF content than progeny from clones with low 6H-DMD values. Progeny from three clone combinations high or low in 6H-DMD values had significantly different NDF, acid detergent fiber (ADF), and acid detergent lignin (ADL) content. The only significant change in cell-wall composition, expressed as percent of the total cell-wall, was lower hemicellulose content of the cell-walls of progeny from clones with high 6H-DMD values. Leaves of two clone combinations with high or low 6H-DMD values had similar NDF content but stems differed significantly in NDF content. Histological studies of the stems revealed greater numbers of xylem cells in progeny with high NDF and low 6H-DMD values.

Medicago falcata clones maintained significant differences in NDF, ADF, and ADL content from early vegetative to full bloom stage of maturity. The relative proportions of cell-wall components, expressed as percent of total cell-wall (NDF), changed very little during ontogeny. The lower calculated digestibility of the final harvest was primarily due to increased cell-wall quantity.

Nutritive evaluations of selected forages were made with weanling meadow voles. Voles fed forages with high 6H-DMD values gained more weight and digested less fiber than voles fed forages with low 6H-DMD values.

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By

John S. Shenk

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INTRODUCTION

The primary objective of the forage breeder is to develop varieties that produce greater quantities of quality feedstuffs for animals. The breeder not only must know the nutritional needs of the animal but also understand the potential and limitation of the specific forage species in supplying the animals nutritional requirements. By integrating these diverse disciplines, the forage breeder has the unique opportunity of producing varieties leading to new levels of nutritional efficiency and economical animal production.

The nutritional quality of alfalfa is superior to most forages and yet high producing animals require supplementary energy for most economical production. The energy supplied by alfalfa, depends upon the relative proportion of cell-wall material and its degree of lignification. The problems of sampling, analysis, and identification of stable plants with superior nutritive value must be overcome in a program designed to improve digestible energy by selection. Chemical and biological procedures are presently available in solving these problems; however, better techniques are needed to obtain an estimation of animal responses in early stages of a breeding program.

Improvement in the digestibility of alfalfa, necessitates genetic manipulation of the cell-wall complex. Changes in cell-wall quantity or composition may be expressed in plant structure and growth characteristics. These relative unknown effects, coupled with alfalfa's differential vegetative response to changing envionmental conditions, requires that the breeder obtain a more thorough understanding of the anatomical functions and physiological production of cell-walls.

The objectives of this study were (1) to evaluate the genetic combining ability of alfalfa clones from two cycles of selection based on high or low six hour *in vitro* dry matter disappearance (6H-DMD) values, and (2) to characterize changes in cell-wall composition that resulted from two cycles of selection. Individual alfalfa genotypes were used to study quantity and composition changes in cell-wall constituents during ontogeny. Studies were also conducted to determine if weanling meadow voles would respond to nutritive differences of selected alfalfa genotypes.

LITERATURE REVIEW

Assessment of Alfalfa Nutritive Value

The high protein, mineral, and vitamin content of alfalfa, coupled with low fiber content are primarily responsible for its superior nutritive value when compared to other forages. The fiber or structural cell-wall constituents and the soluble cellular carbohydrates supply the greatest portion of the potential energy. The kinds and types of carbohydrates in these cell tissues are numerous and complex; nevertheless, a greater proportion of more easily and efficiently digestible carbohydrates is essential if alfalfa quality is to be improved.

Sullivan (1969) discussed the role of forage carbohydrates in ruminant nutrition. The presence and amount of a particular carbohydrate in the forage was probably of less importance than total digestibility of the carbohydrates. The quantity of the cell-wall constituents and degree of lignification were of greater significance than the quantity of more digestible cellular constituents. The high lignin percentage in the cell-wall of alfalfa appeared to be a major deterrent to the release of digestible energy from the cell-wall carbohydrate complex.

Included in the highly soluble cellular constituents were compounds, often attached to simple carbohydrates, which were known to inhibit metabolism and lower nutrient efficiency. These substances and their properties were reported by Pudetkiewicz and Matterson (1960), Smart *et al*. (1961), Hanson *et al*. (1963), Elliott (1963), Shillinger and Elliott (1966), and Jones (1969). Hi Kon Oh *et al*. (1966) stated that rumen *in vitro* techniques often yield more accurate estimates of forage digestibility than chemical methods. This superiority was not surprising because energy releasing bacteria were very likely influenced by unassayed limiting factors active in the substrate (Van Soest, 1969).

Laboratory estimation of forage nutritive value

In vitro procedures have been used for the most part to estimate forage digestibility. Reviews have been written by Barnes (1965), Barnes *et al*. (1964), and Baumgardt *et al*. (1962). The possibility that the nutritive value of a forage can be determined by chemical analysis has been investigated by Van Soest (1964), (1966), (1967), Van Soest and Moore (1965), and Sullivan (1964), (1969). In a few cases small animals have been used to estimate forage nutritive value (Richards *et al.*, 1960; Watson and Horton, 1936; Crampton *et al.*, 1940; Hawkins, 1957; Ingalls *et al.*, 1965). Elliott (1967) believed that the success of a forage breeding program might not depend upon a single analytical procedure but possibly upon a wise selection of complementary laboratory evaluation procedures. These would not only include some of the *in vitro* and chemical techniques but also small animal tests of prospective breeder lines.

Elliott (1963) suggested using the weanling meadow vole as an assay organism in breeding programs for the development of more nutritious forages. The vole is a natural grass eater with a digestive system which is probably capable of gastric and/or caecal digestion of fiber (Hungate, 1966). Daily intake was reported by Hatfield (1935) to be 3.45 grams per day. Studies by Thompson (1965) indicated legumes and adventive grasses were preferred by the vole over native sedges and grasses.

A number of nutritional studies have been conducted for and with the meadow vole. Isolation of antimetabolites from individual alfalfa plants was verified with weanling voles by Schillinger and Elliott (1966). Feeding and management techniques for the vole have been developed by Elliott (1963) and Shenk and Elliott (1969). Weanling voles consumed and grew exceptionally well on high levels of alphacel as the fiber source (Shenk and Elliott, 1968); in addition, a protein-energy ratio of .086 in semi-synthetic diets with 40 to 50 percent alphacel were found to be near optimum for weanling vole growth.

Comparative digestion studies of adult voles with sheep were reported by Cowan *et al.* (1968). The mature vole's digestive system was approximately 78 percent as efficient in fiber digestion as sheep. Cell-wall digestion by the vole has been reported by Keys and Van Soest (1967), and Lynch and Keys (1968).

Alfalfa Plant Organs and Their Relation to Digestion

Plant cell-walls are dissimilar but are grouped according to the tissue they comprise and functions they perform. Seven major cell tissues were described by Fahn (1967). Six of these are important in alfalfa anatomy. The cell-walls of these tissues differ in their chemical composition, structure, and function depending on location in the plant and stage of maturity. In addition, the arrangement and proportions of these tissues differ in the vegetative organs of the plant. These relationships and their implications will be reviewed in the light of current literature on digestion and chemical composition of alfalfa forage.

Alfalfa leaves

Alfalfa leaves contain two basic cell tissues, spongy and palisade parenchyma. These cells have thin primary cell walls; in addition, vascular leaf bundles contain thickened and lignified secondary cell walls. Leaves are formed from primary and axillary meristems differentiating leaf tissue without the addition of new cells during maturation. These anatomical facts support chemical and digestion information noted in this literature review.

Mowat *et al*. (1965) reported that the *in vitro* digestibility of alfalfa leaves decreased only slightly as the plant matured. The decrease was from 78.6 percent to 73.6 percent in Vernal and 77.7 percent to 72.8 percent in DuPuits over a two month period. Terry and Tilley (1964) found alfalfa leaves to be 83 percent digestible on April 2 decreasing to 82 percent on June 25. The lignin content of the leaf was practically constant during this period. Over a period of ten weekly harvests of first growth alfalfa, Barnes and Ferrer (1968) obtained an average of 34.6 percent for acid detergent fiber (ADF) and 6.3 percent for acid detergent lignin (ADL) with a slight increase in these values at maturity.

Macdonald (1946) suggested that the greatest contributing factor in loss of legume nutritive value as maturity was reached was the change in leaf-stem ratio. Leaf content as an indicator of nutritive value was emphasized by Reid *et al*. (1959) and Hillman *et al*. (1968). A high correlation was obtained between the percent leaves in the first cutting and digestible dry matter. Selection for leafiness in alfalfa should increase digestibility of the species and also increase protein content (Mowat *et al*., 1965). Terry and Tilley (1964) postulated that plant breeders should raise digestible dry matter of alfalfa forage by selection for more leafy plants or higher digestibility of the stems.

Alfalfa stems

Young alfalfa stems and leaves were similar in content of cell-wall constituents and extent of *in vitro* digestion. Mature alfalfa stems had a higher cell-wall content and a lower digestibility than corresponding leaves. The upper portion of the stem was higher in digestibility and lower in fiber than the lower stem section (Terry and Tilley, 1964).

The young stem is approximately square with thin walled parenchyma cells extending from just under the epidermis throughout the cortex and into the medullary rays, to the pith (Hayward, 1938; Winter, 1932). Major vascular bundles occur at the corners with smaller bundles interspersed in a ring around the stem. Most of the thickened lignified cell walls are associated with the vascular bundles and related tissue (Wilson, 1913; Winton, 1914). Alfalfa stems averaged 2 percent higher in *in vitro* digestibility than leaves in Terry and Tilley's study (1964) and 2.2 percent to 4.5 percent lower in Mowat *et al.*'s study (1965). Stems from 0-6 inches tall were 79 percent and 77 percent digestible in vegetative and mature plants respectively (Terry and Tilley, 1964).

Stems increase in length and thickness by two meristematic tissues, as the plant matures. The primary meristem adds cell tissue to the apical end of the stem increasing its length and cambial meristems enlarge the vascular tissue and stem diameter by addition of cells (Fahn, 1967). During first growth, a 24 inch increase in stem length resulted in a 37 percent reduction in stem digestibility (Terry and Tilley, 1964). However, no consistent relationship existed between stem width, lignin, or percent ADF content with alfalfa at maturity (Mowat *et al.*, 1966). Vernal and DuPuits stems were similar in digestibility at the same stage of maturity with stem diameter having little influence on digestion. Hayward (1938) reported that secondary thickening of the stem proceeded by way of an intervascular cambium. Meristems developed between the vascular bundles forming a continuous zone of lignified xylem around the stem similar to that of woody tissue. Chemical analysis showed that alfalfa stems averaged 7.5 percent more ADF and 5.9 percent ADL than leaves during the first growth maturation process (Barnes and Ferrer, 1968). In mature Vernal plants, digestibility of the stems was 48.3 percent compared to 73.6 percent for leaves (Mowat et al., 1965). In other digestion trials, in vitro digestibility of mature stems was 26 percent lower than for mature leaves (Terry and Tilley, 1964).

Whole plant

As the plant approaches maturity, digestibility of leaves remains rather constant but stem digestibility decreases rapidly. The ratio of leaves to stems changes from approximately 70 percent at the early vegetative stage to 35 percent at seed set. This is due to at least two factors, leaf loss due to senescence and stem internode elongation increasing the proportions of stems to leaves (Terry and Tilley, 1964).

For these reasons the stem assumes a greater influence on digestibility as the plant approaches harvest-time. As a result the content of indigestible fiber increased and digestible dry matter decreased during ontogeny (Terry and Tilley, 1964). Barnes and Ferrer (1968) reported more than two times as much ADF and ADL in the whole alfalfa plant at seed pod stage as compared to the early vegetative stage.

Environmental factors are known to affect growth and cell-wall production. Kramer (1959) discussed the effects of water stress on plant growth. Water deficits not only reduced total growth, but changed the pattern of growth. Thickness of cell-walls and amount of cutinization and lignification were often increased in plants growing on low moisture levels. Leaf area was usually reduced, but leaf thickness increased. Gifford and Jensen (1967) showed that alfalfa plants grown in compacted soil had reduced yield, higher crude protein content and lower crude fiber levels than plants in average soils. Low soil moisture levels produced the same general effects. Alfalfa had a higher water requirement than other species and composition was more drastically affected by environment than other plant species.

Cell-Wall Properties and Their Influence on Digestion

In herbaceous forage species wood-like tissue of tracheids, vessels and fibers are diluted by other cells whose walls are not strongly lignified. The quantity and composition of these wood-like tissues were the major limiting constituents in extent of forage digestion by rumen microflora. These facts were emphasized by

Johnston and Waite (1965), Sakurai (1963), and Jarrige (1966). Examination of cell debris in the feces of ruminants fed mature forage showed the presence of fibers, occasional intact vascular bundles, and sheets of thickened epidermal cells.

Cell-wall description and developmental control

Cell-walls, as described by Fahn (1967), were unique and varied in composition. Plant tissues composed of primary walls contained interwoven cellulose microfibrils embedded in an amorphous polysaccharide gel. The non-cellulose polysaccharides (hemicellulose) and a small amount of protein composed a large portion of the cell-wall. The walls of parenchyma cells in the cortex, pith, and leaf mesophyll tissue were of this type. Tracheids, vessels, fibers and sclerified xylem parenchyma generally had thick secondary walls with large increases in cellulose and lignification over primary walls. Stafford (1965) discussed the possibility of two-interrelated mechanisms controlling lignification of tissues in timothy: one, the level of caffeic acid ester, the other, the level of 3 indoleacetic acid (IAA).

Cell division and differentiation of cambial cells was stimulated by IAA (Wareing _et_ _al_., 1964). Gibberellic acid (GA) might also be involved in cambium activity;

however, GA alone produced cambial divisions with poor secondary wall formation in xylem. The ratio of IAA to GA regulated the proportion of xylem to phloem in stem tissue (Digby and Wareing, 1966).

Secondary cell-wall composition

Cellulose is the only substance in the cell-wall that is chemically uniform (beta glucosan). The hemicelluloses and pectic substances laid down in conjunction with cellulose are frequently called the matrix materials. Lignin can be called the encrusting substance since it is laid down on or into the existing cell-wall (Clowes and Juniper, 1968). The complexity of the non-cellulose polysaccharide is becoming more apparent. Covalent bonds linking backbone chains of predominantly different sugars are not unlikely. Evidence has been obtained that even cellulose is covalently bonded to sugars other than glucose, presumably a part of the non-polysaccharides.

The lignin encrustation of xylem elements begins at the cell edges, proceeds first into the middle lamella to the primary wall and eventually into the secondary wall. Freudenberg (1965) suggested that in the synthesis of lignin, soluble phenolic precursors were formed in the cambium region which passed to the xylem wall after it was fully secondarily thickened. Lignification then occurred by polymerization of these precursors between the microfibrils of cellulose. Goodman and Siegel (1959) postulated that lignification was a function of the quantity of cellulose in the cell-wall.

The relative indestructible nature of lignin is probably a result of the complex reticulate pattern of heterogeneous linkages; furthermore, lignin has been shown to be covalently linked to cell-wall carbohydrates (Bolker, 1963; Freudenberg and Neish, 1968). A more detailed description of the biochemistry of the lignin formation phenomena has been presented by Bonner and Varner (1965), Clowes and Juniper (1968), and Freudenberg and Neish (1968).

The biochemical mechanism leading to cell-wall formation has been shown by Nevins et al. (1967) to be under genetic control. Significant differences were found in the cell-walls of different species within a given genus. The cell-walls of various morphological parts (roots, hypocotyls, first internodes and primary leaves) of bean plants had a characteristic sugar composition. Sullivan (1966) and Van Soest (1966) have both discussed the digestion of the different proportions of lignin, hemicellulose, and cellulose in grass and legume cell-walls, and their implications. These differences may also be concluded as genetic in origin. Allinson (1966) found different ultraviolet absorption spectra for alfalfa clones of high and low nutritive value, which indicated the possibility of genetic variation for this cell-wall constituent in the *Medicago* species.

Variation in cell-wall composition

Little variation has been reported in the ratios of the three major cell-wall components of mature cells in a species. Thornber and Northcote (1961) stated that although the ratio of increased weight of lignin to alpha-cellulose was constant for each tree species, ratio differences existed among species. Fuller (1958) studied the changes in cell-wall components of bean roots during development. In the region from 43 to 60 mm. from the root tip, where differentiation was complete, little change occurred in the average cell-wall composition. Hardwick (1954) stated that percentages of the structural carbohydrates, particularly cellulose and hemicellulose, remained fairly steady from germination to flowering in subterranean clover. The ratios of hexose, pentose, and lignin in the cell-walls of alfalfa stems harvested in the early vegetative stage were almost the same as those obtained from mature alfalfa stems (Terry and Tilley, 1964). In a study by Barnes and Ferrer (1968) the lignin as a percent of the total acid detergent fiber increased by 2 percent from young to mature stems while total production of these components doubled.

Digestion of cell-wall components

Cell-wall properties and their relation to microbial digestion have been discussed by Bonner and Varner (1965). Cell-wall polysaccharides might be highly specific molecules existing in unique three dimensional structures depending on their composition. An alteration in the ratios of sugars which compose a polysaccharide could result in a change in susceptibility to enzymatic attack. Variation in sugar composition might result from alteration of the ratio of sugar nucleotides available for synthesis, or changes in the levels or specificities of enzymes which catalyze the synthetic reaction. In this way, a quantitative alteration in cell-wall polysaccharides could affect the rate of microbial breakdown.

Lignification is the most important factor affecting digestion of forage plants. The ratios of lignin to other cell-wall components has been shown to limit or inhibit cell-wall digestion. Van Soest (1967) suggested using a summative equation in which the lignin-acid detergent fiber ratio was used to estimate the true digestibility coefficient of the cell-wall. Sullivan (1966) concluded that lignin affected the digestion of hemicellulose more than it influenced the digestion of cellulose in alfalfa. Lignin was postulated to replace hemicellulose as the plant becomes more mature because of greater chemical linkage of lignin to hemicellulose.

Other cell-wall properties have been shown to limit digestibility of forages. Van Soest (1969) stated that crystallinity has not been successfully related to digestibility. More crystalline structures should be more resistant to digestion. Individual sugar residues have shown digestibility differences. Xylose was consistently less digestible than arabinose (Jarrige, 1961; Gaillard, 1962; and Lyford *et al*., 1963). Uronic acid seemed to be the lowest in digestibility and should possibly be considered a negative index of nutritive value (Gaillard, 1966). Elliott (1967) has speculated that the poly-phenol lignin precursors and degradation products of digestion belong in this category. Although lignin is probably the major determinant of forage digestibility, other cell-wall constituents may modify or enhance its effects.

Breeding for Improved Digestibility

The literature concerning breeding for improved forage digestibility covers a wide range of cross-fertilized perennial species. Elliott (1958) stated that the evolutionary forces of natural selection have acted primarily on the vegetative phases of growth. The more a phenotype withstands environmental variation by buffering against this variation, the less effective artificial selection may be. Progress in selection for a quantitative character like in vitro digestion may be slow since digestibility is closely related to the structural composition of the vegetative tissue.

Allinson (1966) screened different varieties and species of individual alfalfa plants and reported a large range in values for six hour in vitro dry matter disappearance (6H-DMD). Population means were significantly different and a negative correlation was found between 6H-DMD values and yield in grams per plant. DuPuits and Vernal clones selected for high 6H-DMD were lower in NDF, ADF, and ADL content. Volatile fatty acid production in an in vitro incubation was consistently higher from those clones that had high 6H-DMD values. Clones with superior nutritive values gave different ultraviolet absorption spectra than those having low 6H-DMD values. A greater percentage of the total lignin was extractable from clones of high nutritive value than from those of low nutritive value. Allinson suggested that control of lignin development in the forage crop could infinitely improve forage crop utilization.

Studies with the six hour dry matter disappearance technique were continued by Shenk (1967). Environmental influence and interaction altered the 6H-DMD values of spaced plants of alfalfa clones. The agronomic characters of yield as well as disease and insect resistance were not necessarily related to values for 6H-DMD in all populations. Shenk stated that preliminary screening of large numbers of individual plants could be effectively carried out with the 6H-DMD procedure by making only one determination per sample.

A study of the combining ability of *in vitro* digestibility of alfalfa was conducted by Gil *et al*. (1967). Significant general and specific combining abilities were found for the parental clones studied. Negative correlations between yield and *in vitro* dry matter digestibility (IVDMD) (Tilley and Terry procedure, 1963) were found. The ten crosses with the highest specific combining effects for IVDMD had negative specific combining effects for yield. Of the ten crosses with the highest specific combining effects for yield, three had positive specific combining effects and seven had negative specific combining effects for IVDMD. The authors concluded that selection for yield would increase the total nutrient production per acre and therefore may be more profitable than selection for high *in vitro* digestibility.

Burton *et al*. (1967) improved the digestibility of bermudagrass by breeding and selection. Leaf percentage was not a good index of digestibility for the genotypes

utilized. Knight and Yates (1968) found environmental influences confusing the digestibility expression of cocksfoot genotypes. Within a harvest, general combining ability and parental performance were good; however, little uniformity in ranking of parental clones or their progeny occurred from one harvest to the next. Knight concluded that selection for a specific group of genotypes uniformly high in digestibility over cuttings would be difficult. The reasons for these genotype discrepencies were unknown, but were not believed due to growth rhythm.

Finally Carlson et al. (1969) reported the same general findings in IVDMD of fall-saved reed canarygrass. Significant parent-progeny correlations indicated heritable variation in IVDMD. Highly significant clone x year interactions indicated selection should be based on more than one years result. All correlations with yield were negative and those with percent crude protein positive. Selection on clone performance was postulated as being effective for improving the digestibility trait; however, tests should be based on mean performance in replicated tests conducted for at least two years.

MATERIALS AND METHODS

Plant Sources and Evaluation Procedures

Alfalfa clones used in this study originated from three *Medicago* species, *Medicago sativa* (varieties Vernal, Culver, DuPuits; USDA lines MSA-C4, and MSB-C4), *Medicago glutinosa* (Russian source 29003), and *Medicago falcata* (Russian source 258751).

The first cycle included clones from unselected populations Culver, Vernal, MSA-C4, MSB-C4, and *M. glutinosa*, and progeny from Vernal and DuPuits clones selected by Allinson (1966) on the basis of *in vitro* dry matter disappearance (6H-DMD) values. These clones were transplanted as seedlings into the field in the spring of 1965. Harvests were made June 10 and July 30, 1967. Characters evaluated were 6H-DMD and clone dry weight.

General and specific combining abilities were calculated with these variables for six Vernal clones high in 6H-DMD. Fifteen crosses, or one half of the complete diallel, were evaluated with eight progeny from each cross. Ten crosses were harvested in bulk and expressed as mean values for the combination in the diallel table. Eight clones of each of the remaining five crosses were individually harvested and used to estimate the within combination variance.

Clones with high or low 6H-DMD values were selected from each of the source populations in the first cycle. First and second cutting forages of these clones were analysed for cell-wall constituents (NDF), acid detergent fiber (ADF), and acid detergent lignin (ADL). Five of these clones were also analysed with the <u>in vitro</u> procedure of Tilley and Terry (1963). Nutritive evaluation of selected forages were made with weanling meadow voles.

Second cycle progeny generated from selected first cycle clones of high x high and low x low in 6H-DMD values were transplanted as seedlings into the field in the spring of 1968 and harvested June 11, 1969. Ten individual progeny from each cross were evaluated for dry weight, percent leaves, and NDF. Leaf percentages and plant height were estimated for each clone from a single representative stem. Griffing's Model 1, Method 4 (1956) was used to partition mean squares for crosses into general and specific combining abilities for clones in both first and second cycles.

Progeny from three clone combinations with high and three clone combinations with low 6H-DMD values were analysed for ADF and ADL. Leaves and stems of two clone combinations (B75 x G23-12 high in 6H-DMD (H $\frac{B}{G}$), and

V232/219-2 x D69/D52-9 low in 6H-DMD $(L \frac{V}{D})$ were analysed for NDF, ADF, and ADL. Histological sections were made from stems of five representative clones in each of these two clone combinations.

Asexually reproduced M. falcata clones were transplanted into the field in the spring of 1965 and harvested three years later at ten day intervals between May 4 and July 4. The only characters evaluated in each harvest were NDF, ADF, and ADL.

All clones in each study were planted on .91 m. centers, harvested at first bloom (with the exception of M. falcata clones), dried at 43-47 C., ground through a 1 mm. screen, and stored at 0.6 C.

Cell-Wall Analysis

Neutral detergent fiber (NDF), acid detergent fiber (ADF), and acid detergent lignin (ADL) values were determined according to the procedures of Van Soest (1962) and Van Soest and Marcus (1964). Second cycle progeny were not analysed in duplicate. Estimates of the percent hemicellulose, and cellulose were obtained by subtraction (NDF minus ADF equals hemicellulose, ADF minus ADL equals cellulose). These two cell-wall components and lignin (ADL) were expressed as percent of the total cell-wall (NDF). These calculations were used to express composition changes in cell-walls of second cycle progeny and *M. falcata* clones.

Rumen In Vitro Procedures

Forage samples were analysed by the six hour *in vitro* dry matter disappearance procedure outlined by Allinson (1966). This procedure was based on the work of Bowden and Church (1962), Baumgardt *et al.* (1962), and modifications by Ingalls (1964). Only single determinations using one gram were run because of the relatively small standard deviation between duplicate samples (Shenk, 1967). A standard forage was included in each fermentation batch to correct for differences among fermentations. A two stage *in vitro* procedure, Tilley and Terry (1963), was used to analyse first and second cuttings of five selected first cycle clones.

Histological Procedures

Stems were chosen from five representative progeny of two combinations $H \frac{B}{G}$ and $L \frac{V}{D}$, at the first bloom stage of maturity. The second or third internode from the crown of the plant was selected in such a way that a cross section, 4 mm. in diameter near the center of the internode, could be taken five inches from the soil level. The entire internode was placed in a fixing solution (50 ml. 95 percent alc., 5 ml. glacial acetic acid, 10 ml. formalin, 35 ml. water), and sectioned without supplementary support with a microtome (Hooker, 1967). Staining techniques were carried out according to the safranin-fast green procedure outlined by Johansen (1940).

The number of cells in the radius of the xylem were obtained by counting from the cambium to the last red cell-wall in the pith. The number of cells were counted at a circumference in the middle of the xylem ring. Ten radii and two circumference counts were made under 100 X. magnification for each stem.

Weanling Vole Assay

Seventeen litters of uniform size and condition were chosen for these studies. Litters were reduced to five or six individuals eight to ten days after birth. Twelve to 14 days after birth litters were weaned and placed on an intermediate diet (Table 1) for 24 to 48 hours. When started on test diets, mean weight of weanlings was 11.8 ± .83 grams. Sex was not determined. All weanlings in the litters were randomly assigned to diets in each experiment and placed in laboratory nests, bedded with San-i-cel, or placed on wire screens for feces collection. Length of feeding period in each experiment was six days.

The environmental conditions of light, temperature, and watering procedures outlined by Elliott (1963) were

Table 1.--Weanling vole diet ingredients and formulations expressed in percent.

Ingredient	Intermediate Diet	Exp. 1	Exp. 3	
			Low[f]	High[f]
Casein[a]	20	--	--	--
Alfalfa	15	78	48	83
Butter	6	--	--	--
Carbohydrate Mix[b]	52	17	45	10
Corn Oil	2	--	--	--
Vitamins[c]	2	2	2	2
Mineral[d]	3	3	3	3
Cellulose Gum[e]	--	--	2	2

[a] "Vitamin free" casein, 14.5 percent nitrogen, Nutritional Biochemicals Corporation, Cleveland, Ohio.

[b] Carbohydrate mix consisting of 50 percent corn starch, 25 percent dextrin, 25 percent sucrose.

[c] Vitamin Diet Fortification Mixture, Nutritional Biochemicals Corporation, Cleveland, Ohio.

[d] Salt Mixture W, Nutritional Biochemicals Corporation, Cleveland, Ohio.

[e] Cellulose gum, type 7HF, Hercules Inc., Wilmington, Delaware.

[f] Low refers to diets consisting of 48 percent alfalfa. High refers to diets consisting of 83 percent alfalfa.

followed throughout the experimental period. Feces were collected and analysed for NDF according with the procedures of Van Soest and Marcus (1964).

Experiment 1

A randomized design was employed in this experiment. A total of 30 weanlings were fed eight forages with unequal replication. Diets contained 78 percent dietary alfalfa, Table 1, and were fed according to the procedures of Shenk and Elliott (1969). Correlation coefficients were calculated between 6H-DMD values and corresponding weanling weight gains.

Experiment 2

In order to obtain accurate measurements of intake with the procedures outlined by Shenk and Elliott (1969), diets should be in the form of a non-crumbling wafer. This is necessary to prevent errors in the weighing procedure caused by voles spilling wasted diet from the feeder. Alfalfa wafers with low levels of added carbohydrate mix need additional binding agents to prevent excessive diet waste. Gums and adhesives were added to the diet wafers to test their capacity to alleviate the waste problems.

1. Ten adhesives were tested in alfalfa wafers: gum arabic, acacia gum, sorbitol, locus bean gum, cellulose gum, Kelcoloid LV, B-glucan, guar gum,

crystalline cellulose, and Kelcoloid HV. All adhesives were added to the diet formulation of experiment 1 at 2 percent.

2. Two adhesives were incorporated into semi-synthetic diets (14 percent protein, 41 percent carbohydrate mix, 40 percent alphacel, and 5 percent mineral and vitamins) at 2 percent and 5 percent. A randomized block design was used involving 15 weanlings. Waste comparisons were made on a percent basis between the control and treated diet means.
3. Alfalfa diets were mixed according to the formulation of experiment 1, with the addition of 2 percent of the selected adhesive. Diets were fed to six voles in a paired design. Waste comparisons were made on a mean percent basis between the treated and untreated diets.

Experiment 3

Forages from Vernal and DuPuits crosses designated as high or low in 6H-DMD values were composited and fed at 48 and 83 percent of the diet, Table 1. Three high 6H-DMD clones from MSB-C4 and three MSA-C4 clones low in 6H-DMD, were bulked. Only first cutting forages were used. A partially nested design was used to assign diets to eight litters with a total of 30 weanlings. Six litters of four weanlings each were randomly assigned to two alfalfa levels of high and low Vernal and USDA lines (MSB-C4 and MSA-C4) 6H-DMD composites. Two litters were fed DuPuits composites in like manner. At the end of each six day feeding trial the entire nest was dried and sieved through a 1/2 mm. screen.

RESULTS

First and Second Cycle of Selection in Alfalfa for 6H-DMD

First cycle analysis

Progeny analysis of selected Vernal and DuPuits clones.--Statistics are presented in Table 2 for the progeny of Vernal clones which had been selected for high and low 6H-DMD values. The progeny from the clones high in 6H-DMD had a mean value of 36.9 percent in the first cutting and 41.3 percent in the second. In contrast the progeny from two clones low in 6H-DMD had a mean of 34.4 percent and 37.5 percent for the respective cuttings. Error mean squares were of the same relative magnitude in both cuttings and populations. The progeny mean of selected clones high in 6H-DMD was 2.5 and 3.8 actual percent higher in *in vitro* dry matter disappearance in first and second cuttings than the progeny from clones low in 6H-DMD.

Combining ability analysis was conducted on the six clones high in 6H-DMD. Mean squares for general combining ability for 6H-DMD were significant in the first

Table 2.--Combining ability analysis of first cycle Vernal clones high in 6H-DMD. Ranges, means, mean differences, and mean squares for 6H-DMD and yield of both high and low progeny. Values for 6H-DMD are expressed in percent and yield values are expressed in grams.

				Mean Squares		
	Range	Mean	Diff.[c]	gca.[f]	sca.[g]	Error
High Prog.[a]						
6H-DMD[d]	34.4-40.0	36.9	+2.5**	2.68*	1.06	.89
6H-DMD[e]	37.2-46.2	41.3	+3.8**	4.23**	2.67**	.37
Yield[d]	71-258	131.9	+33.0**	320.00	347.50	263.40
Yield[e]	17-113	48.7	+21.4**	302.90**	131.90**	41.60
Low Prog.[b]						
6H-DMD[d]	31.2-37.7	34.4				.41
6H-DMD[e]	34.4-41.6	37.5				.52
Yield[d]	61-158	98.9				123.90
Yield[e]	14-60	27.3				17.40

[a] Progeny analysis of six parental clones selected for high 6H-DMD values. Total Progeny 120.

[b] Progeny analysis of two parental clones selected for low 6H-DMD values. Total progeny 20.

[c] Difference between means of progeny from parental clones high or low in 6H-DMD values.

[d] Date of harvest June 10, 1967.

[e] Date of harvest July 30, 1967.

[f] General combining ability.

[g] Specific combining ability.

*Significant $P < .05$; **significant $P < .01$.

and second cutting. Specific combining ability in the second cutting was also significant. General and specific combining abilities were not significant for yield in the first cutting. Calculation of general combining effects for the six parental clones indicates only one clone had positive general combining effects for 6H-DMD and yield, Table 3.

Table 3.--Estimates of general combining effects of Vernal clones for 6H-DMD and yield.

Clones	6H-DMD[a]	6H-DMD[b]	Yield[b]
V7	-1.41	-1.02	+1.10
V214	-.46	+.81	-9.60
V132	+.59	+.96	+16.10
V51	+.09	-.46	-4.63
V46	+.37	-1.24	-1.83
V37	+.82	+.96	-1.15
S.E. $(\hat{g}_i - \hat{g}_j)$	.66	.44	4.48

[a] Date of harvest June 10, 1967.

[b] Date of harvest July 30, 1967.

The Vernal clones high in 6H-DMD had high yield potential. Their progeny outyielded the progeny from Vernal clones low in 6H-DMD in both cuttings. The second cutting had lower yield and higher 6H-DMD values than the first, but differences in 6H-DMD values between progeny of clones for both cuttings were consistent.

Statistics for 6H-DMD and clone yield for progeny of selected high and low DuPuits clones are presented in Table 4. The means of the progeny from two clones high in 6H-DMD were 36.1 percent and 40.2 percent in the first and second cuttings respectively. The progeny means from the three clones low in 6H-DMD were 32.6 percent and 39.7 percent in each cutting. The progeny from selected clones high in 6H-DMD were 3.5 and 0.5 actual percent higher in 6H-DMD than the progeny from clones low in 6H-DMD.

The mean yield of the progeny from clones low in 6H-DMD was significantly higher than the progeny of clones high in 6H-DMD, but no differences existed in mean yields of second cuttings among the two progeny groups. These results are the reverse to those reported for the Vernal progeny. Six hour DMD values appear to be closely related to changes in clonal yields in this population.

Screening of new populations for clones high and low in 6H-DMD values.--Five populations of unselected clones, MSA-C4, MSB-C4, Vernal, Culver, and *M. glutinosa* were screened for clones high and clones low in 6H-DMD. The 6H-DMD character was normally distributed in each of these populations. Mean differences among populations were significant in the first cutting, Appendix, Table 19.

Table 4.--Progeny evaluation of selected clones of DuPuits. Ranges, means, mean differences, and standard deviations for 6H-DMD and yield of both high and low progeny. Values for 6H-DMD are expressed in percent and yield values are expressed in grams.

	Range	Mean	Diff.[c]	SD[d]
<u>High Prog.</u>[a]				
6H-DMD[e]	33.1-44.2	36.1	+3.5**	3.00
6H-DMD[f]	36.1-45.5	40.2	+0.5	2.45
Yield[e]	11-107	67.8	-30.8**	26.60
Yield[f]	20-70	45.3	-0.4	13.10
<u>Low Prog.</u>[b]				
6H-DMD[e]	29.8-35.4	32.6		1.83
6H-DMD[f]	35.9-41.9	39.7		1.26
Yield[e]	51-163	98.6		29.20
Yield[f]	25-81	45.7		13.10

[a] Progeny analysis of two parental clones selected for high 6H-DMD values. Total progeny 18.

[b] Progeny analysis of three parental clones selected for low 6H-DMD values. Total progeny 27.

[c] Differences between means of progeny from parental clones high or low in 6H-DMD values.

[d] Standard deviation.

[e] Date of harvest June 10, 1967.

[f] Date of harvest July 30, 1967.

**Significant $P < .01$.

A range of 10 percent or better in 6H-DMD values existed within cuttings of each population. First cutting results were similar to those reported by Allinson (1966) and Shenk (1967). Population mean values of the second cutting were more nearly alike but from 3.6 to 9.4 actual percent higher than the values for second cuttings. Standard deviations for 6H-DMD values were of similar magnitude among cuttings and populations. These general trends are similar to those reported for the Vernal and DuPuits progeny. Only the MSB-C4 population had a consistently high 6H-DMD mean in both first and second cutting relative to other populations.

Mean dry weight yields of individual clones differed somewhat among populations in both cuttings. Large ranges in clonal yields were observed. The greatest yield differences occurred as reductions of all clonal yields from first to second cutting. This decrease in mean dry weight was accompanied by a reduction in the standard deviation as in the Vernal and DuPuits progeny.

Population mean squares for 6H-DMD and clonal yield values are presented in the Appendix, Table 20. Highly significant cutting effects existed in all populations for 6H-DMD. Clonal means were either nonsignificant or significant at low levels of probability. Cutting and clonal mean squares were highly significant for the yield character.

Relationships between in vitro 6H-DMD and clonal dry weight.--Correlation coefficients between 6H-DMD and clonal dry weight values are presented in Table 5. All correlations were negative or near zero in both cuttings. In general the log transformation of weight increased the correlation coefficients for first cutting values, indicating a curvilinear relationship between 6H-DMD values and clonal yield.

Analysis of variance of four DuPuits crosses revealed a significant cutting x cross interaction for both 6H-DMD and yield, Appendix, Table 21. These results are summarized in Figure 1.

All means of crosses for 6H-DMD increased on second cutting and all yield means for crosses decreased on second cutting. In the first cutting those crosses with high 6H-DMD means had low mean yields and *vice versa*. Results of second cutting demonstrate the changes responsible for the significant interaction. The highest yielding crosses in the first cutting had the greatest second cutting yield reduction and the largest increase in 6H-DMD values; likewise, the lowest yielding crosses in the first cutting had the smallest reduction in yield in the second cutting and consequently the smallest increase in 6H-DMD values.

Table 5.--Correlation coefficients between clone yield and 6H-DMD values for first and second cuttings in all populations.

	First Cutting		Second Cutting	
Population	(1)	Log.[a]	(2)	Log.[a]
Vernal High	-.32*	-.38*	.05	-.01
Vernal Low	.01	.07	-.32	-.01
DuPuits High	-.73**	-.80**	-.46*	-.46*
DuPuits Low	-.31	-.36	-.22	-.26
MSA-C4	-.27	-.36*	-.19	-.20
MSB-C4	-.22	-.31*	.03	.01
Vernal	-.29	-.29	-.70**	-.69**
Culver	.04	.08	-.11	-.14
Glutinosa	-.39**	-.47**	-.33*	-.34*

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[1] Correlation coefficient between first cutting yield and first cutting 6H-DMD values.

[2] Correlation coefficient between second cutting yield and second cutting 6H-DMD values.

[a] Logarithm of weight value correlated with respective 6H-DMD value.

*Significant P < .05; **significant P < .01.

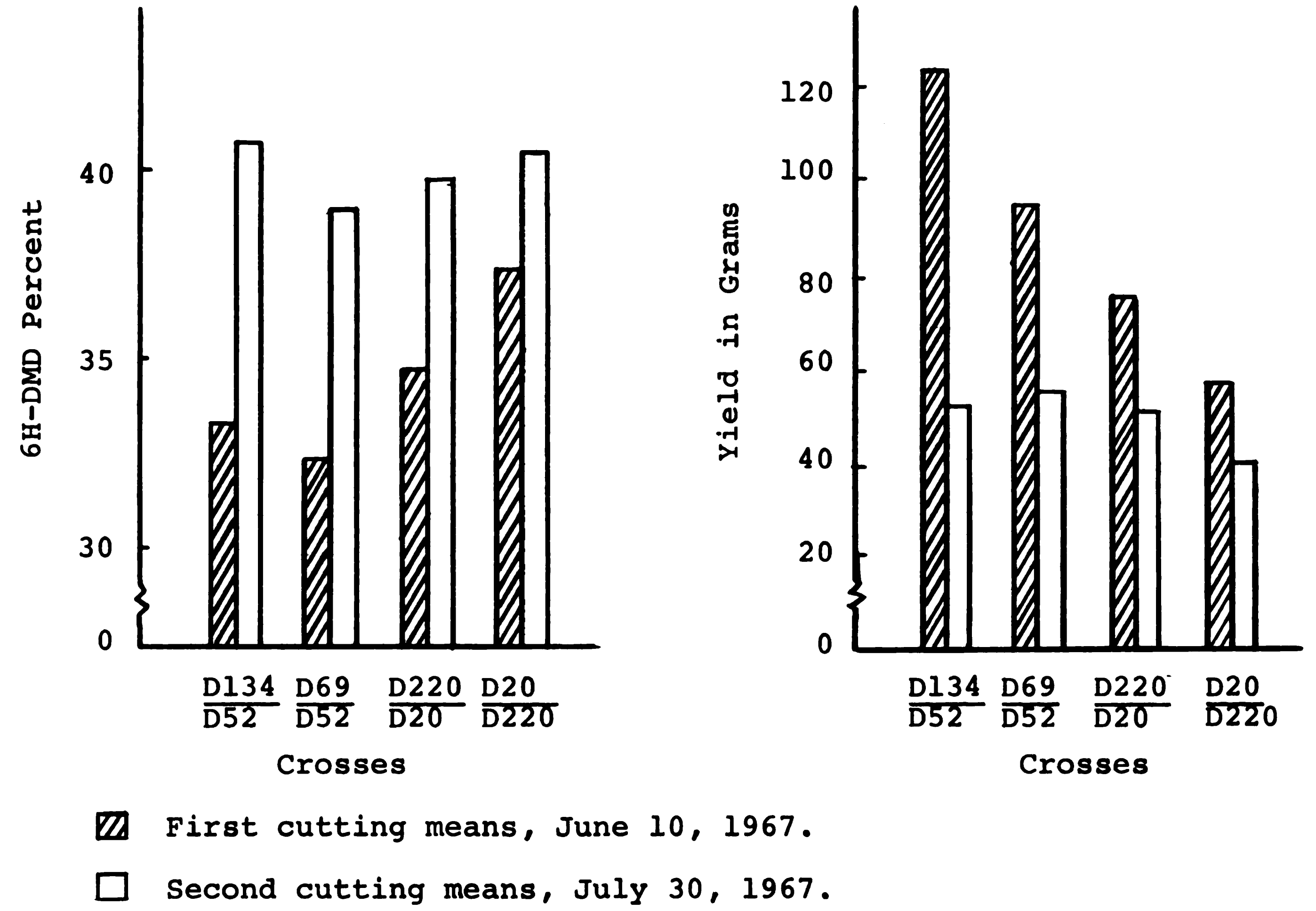


Figure 1.--Graph of cutting times clone interaction for four DuPuits crosses.

Cell-wall analyses of selected clones.--The forage from a clone high and a clone low in 6H-DMD was selected for chemical analysis from progeny of Vernal and DuPuits, and from MSA-C4, MSB-C4, and *M. glutinosa* populations. Actual *in vitro* and chemical data are presented in the Appendix, Table 22. With only one exception, NDF and ADF content was higher for clones selected for low 6H-DMD than the corresponding clones high in 6H-DMD from each population. Content of NDF and ADF in the second cutting was generally lower than the first cutting. Values for ADL were not as consistently different. Clones low in 6H-DMD had higher ADL content than the respective clones high in 6H-DMD in both first and second cutting. Six out of ten clones had higher ADL content for second cutting than first.

Correlation coefficients among these variables are presented in Table 6. The log transformations of yield were negatively correlated with *in vitro* values and positively correlated with NDF content. Neither of these correlations were significant. Content of ADF and NDF was negatively and significantly correlated with both *in vitro* fermentation values. Neutral detergent fiber content was highly correlated with ADF and ADL content. The ADL/ADF ratio was negative but not significantly correlated with either *in vitro* fermentation value. Correlation

Table 6.--Correlation coefficients for cell-wall components and *in vitro* fermentation values of selected alfalfa clones.

Variable	TT[a]	6H-DMD[b]	NDF[c]
Yield[d]	-.30	-.42	.41
ADL[e]	-.56	-.67**	.81**
ADF[f]	-.74*	-.79**	.93**
NDF	-.89**	-.84**	--
ADL/ADF	-.05	-.09	.15
Van Soest Summative Equation	.70*	.73**	--
6H-DMD	.85**	--	.84**

[a] Tilley and Terry procedure, 8 df; all other correlations 18 df.

[b] Six hour dry matter disappearance procedure.

[c] Neutral detergent fiber.

[d] Logarithm of yield value correlated with respective *in vitro* value.

[e] Acid detergent lignin.

[f] Acid detergent fiber.

*Significant $P < .05$; **significant $P < .01$.

coefficients similar to these were reported by Allinson (1966). The correlation coefficients between digestibility, calculated from Van Soest's summative equation and *in vitro* fermentation values, were positive and significant although smaller than the correlation between *in vitro* and NDF values.

Second cycle analysis

Second cycle selection and breeding procedures.--Clones high and low in 6H-DMD were selected from each first cycle population. Vernal clones high in 6H-DMD were selected from the cross V37/V132 which was highest in 6H-DMD. These clones had positive general combining effects for 6H-DMD in both first and second cutting, although only V132 had positive general combining effects for yield. Vernal clones low in 6H-DMD were selected from the V232/V219 cross. The clones of DuPuits which were highest in yield were selected from the combination D20/D220 and high yielding clones from the cross lowest in 6H-DMD, D69/D52.

From the remaining five individual clonal populations, clones were selected for the second cycle if they had 6H-DMD values in the upper or lower 10 percent of the population in each cutting. A second restriction was used in that selected clones had to have at least average yield values in their respective populations. Final choice of six clones high in 6H-DMD and four clones low in 6H-DMD was a matter of convenience related to flowering and seed setting under greenhouse conditions.

Combining ability analysis of selected clones from first cycle.--The statistics for progeny from the selected second cycle clones both high and low in 6H-DMD are presented in Table 7. Three characters were measured in these two populations and significant mean differences were obtained. The progeny of the selected clones high in 6H-DMD were 3.32 actual percent lower in NDF content, 7.15 actual percent higher in leaves, and 19.68 grams higher in dry weight.

General and specific combining abilities were calculated for clones both high or low in 6H-DMD. With the exception of specific combining ability for percent leaves in the clones selected for low 6H-DMD, all general and specific combining abilities were significant at 1 percent. Error mean squares for variables in each population were of similar magnitude.

The general combining effects were calculated for each parental clone, Table 8. Of the six clones high in 6H-DMD four had negative general combining effects for NDF content. These four clones had positive combining effects for percent leaves, and two had positive combining effects for yield. From these results, progeny from the

Table 7.--Combining ability analysis of second cycle clones high or low in 6H-DMD. Ranges, means, mean differences, and mean squares for progeny in percent leaves and NDF, and yield. Values for NDF are expressed in percent and yield values expressed in grams.

				Mean Squares		
	Range	Mean	Diff.[c]	gca.[e]	sca.[f]	Error
High Prog.[a]						
NDF[d]	30.66-43.53	37.49	-3.32**	6.58**	1.88**	.44
Leaves	35.33-59.86	45.44	+7.15**	6.12**	7.92**	1.64
Yield	51-235	131.15	+19.68*	910.05**	274.76**	70.64
Low Prog.[b]						
NDF[d]	35.99-48.75	40.81		5.22**	17.16**	.42
Leaves	26.32-48.00	38.29		30.52**	2.45	.92
Yield	55-192	111.47		732.57**	516.60**	98.24

[a] Progeny analysis of six parental clones selected for high 6H-DMD values. Total progeny 150.

[b] Progeny analysis of four parental clones selected for low 6H-DMD values. Total progeny 60.

[c] Differences between means of progeny from parental clones high or low in 6H-DMD values.

[d] Neutral detergent fiber.

[e] General combining ability.

[f] Specific combining ability.

*Significant $P < .05$; **significant $P < .01$.

three clone combination of B, G, and D were selected for more detailed chemical analysis. Clone D was chosen over C because D had a larger combining effect for yield.

Table 8.--Estimates of general combining effects for percent leaves and NDF, and yield of clones high in 6H-DMD.

Clones[b]	NDF[a]	Leaves	Yield
C	-.09	+.87	-16.47
G	-.42	+.25	+5.71
LV	+1.46	-2.48	-13.12
B	-1.66	+.45	+5.13
V	+1.56	+.20	+24.54
D	-.85	+.71	-5.79
S.E. $(\hat{g}_i - \hat{g}_j)$	.47	.91	5.94

[a] Neutral detergent fiber.

[b] C equals C162/C108-3, G equals G23-12, LV equals H5/L4-4, B equals B75, V equals V37/V132-5, D equals D20/D220-2.

Table 9 contains the specific combining effects and means of crosses for the six clones high in 6H-DMD. The four crosses with the lowest specific combining effects for yield had negative specific combining effects for NDF content. Likewise, the four crosses with the highest specific combining effects for yield had positive specific combining effects for NDF content.

Table 9.--Estimates of specific combining effects for percent leaves and NDF, and yield of each cross from clones high in 6H-DMD, and mean of crosses.

Parent Clone	Trait	Parent Clones					$\bar{X}$ Crosses
		G	LV	B	V	D	
C	NDF[a]	-.52	-.04	-.27	+2.64	-1.81	37.39
	Leaves[b]	+1.54	-1.54	-2.14	-2.42	+4.57	46.13
	Yield[c]	-28.80	-4.40	+5.80	+20.90	+6.30	117.98
G	NDF		-.53	+.79	-.84	+1.10	37.13
	Leaves		-.76	-1.64	+.38	+.50	45.64
	Yield		-.10	+15.80	+1.30	+11.90	135.72
LV	NDF			+.28	-1.08	+1.37	38.63
	Leaves			+1.41	+1.22	-.35	43.46
	Yield			-2.90	-6.00	+13.30	120.66
B	NDF				-.43	-.36	36.14
	Leaves				+3.96	-1.57	45.79
	Yield				-1.90	-17.00	135.26
V	NDF					-.29	38.72
	Leaves					-3.14	45.60
	Yield					-14.40	150.78
D	NDF						36.79
	Leaves						46.00
	Yield						126.52

[a] Neutral detergent fiber. S.E. difference between two crosses having no parent in common equals 0.82; having one parent in common equals 0.54.

[b] S.E. difference between two crosses having no parent in common equals 1.57; having one parent in common equals 1.04.

[c] S.E. difference between two crosses having no parent in common equals 10.29; having one parent in common equals 6.86.

Two of the three crosses chosen for further analysis, B/G and D/G, had positive specific combining effects for NDF content and yield whereas these same effects were negative for D/B. Only D/G had positive specific combining effects for percent leaves. The mean NDF content, percent leaves, and yield of the three crosses was 36.02 percent, 45.46 percent and 138.07 grams respectively. Content of NDF was the only variable significantly lower than the progeny means of the six clones.

General combining effects for the four clones low in 6H-DMD are presented in Table 10. The Vernal clone (V)

Table 10.--Estimates of general combining effects for percent leaves and NDF, and yield of clones low in 6H-DMD.

Clones[b]	NDF[a]	Leaves	Yield
C	-1.61	+4.64	+6.90
D	-.38	-2.66	-26.60
V	+2.24	-3.74	+18.50
B	-.25	+1.76	+1.20
S.E. $(\hat{g}_i - \hat{g}_j)$	.65	.96	9.91

[a] Neutral detergent fiber.

[b] C equals C13/C5-1, D equals D69/D52-9, V equals V232/V219-2, B equals B9.

had positive general combining effects for NDF content and yield, and negative general combining effects for percent leaves. The B and D clones had negative general combining effects for NDF content but only the DuPuits clone had negative combining effects for yield. The Culver clone (C) had negative combining effects for NDF content and positive general combining effects for percent leaves. For these reasons, the progeny of the Culver clone were dropped from further chemical analysis.

Specific combining effects for crosses of the four clones low in 6H-DMD are presented in Table 11. Results were difficult to interpret because of the symmetry of the effects calculated with only four parents.

The correlation between clonal yield and NDF content was positive in each population, Table 12. This supports the findings of Allinson (1966) and those of the first cycle analysis. The correlation coefficients between clone height and NDF content were of greater magnitude than yield-NDF correlations, but only among progeny of all clones high or low in 6H-DMD. Correlation coefficients for height-leaves and leaves-NDF relationships were negative and significant.

Complete cell-wall analysis of progeny from selected three clone combinations.--The progeny from the three clones high and three clones low in 6H-DMD values, selected from the second cycle, were analysed for ADF and ADL content. Correlation coefficients among variables evaluated for these progeny are found in Table 12. The correlation coefficients between NDF and ADF contents were positive and highly significant in each population. All other correlations for cell-wall components were positive but varied in their degree of significance.

Table 11.--Estimates of specific combining effects for percent leaves and NDF, and yield of each cross from clones low in 6H-DMD, and mean of crosses.

Parent Clones	Trait	Parent Clones			$\bar{X}$ Crosses
		D	V	B	
C	NDF[a]	+.09	-1.75	+1.67	39.74
	Leaves	--	--	--	41.39
	Yield	-1.00	-15.60	+16.50	116.07
D	NDF		+1.67	-1.75	40.56
	Leaves		--	--	36.52
	Yield		+16.50	-15.60	93.73
V	NDF			+.09	42.31
	Leaves			--	35.80
	Yield			-1.00	123.80
B	NDF				40.64
	Leaves				39.47
	Yield				112.27

[a] Neutral detergent fiber. S.E. difference between two crosses having one parent in common equals 0.65.

[b] S.E. difference between two crosses having one parent in common equals 9.91.

Table 12.--Correlation coefficients among cell-wall constituents, percent leaves, yield, and clone height for progeny of all clones high or low in 6H-DMD, and selected three clone combinations of clones high or low in 6H-DMD.

Correlation	High[a]	Low[b]	High[c]	Low[d]
Yield - Height	.19*	.26*	.26	.41*
Yield - NDF[e]	.37**	.40**	.54**	.73**
Yield - Leaves	-.08	-.10	-.25	-.44*
Height - NDF	.42**	.57**	.18	.54**
Height - Leaves	-.56**	-.68**	-.64**	-.73**
Leaves - NDF	-.43**	-.57**	-.23	-.60**
NDF - ADF[f]	--	--	.84**	.53**
NDF - ADL[g]	--	--	.29	.12
ADL - ADF	--	--	.21	.45*

[a] Correlations among variables for progeny of six clones high in 6H-DMD values, df equals 148.

[b] Correlations among variables for progeny of four clones low in 6H-DMD values, df equals 58.

[c] Correlations among variables for progeny of three clones high in 6H-DMD values, df equals 28.

[d] Correlations among variables for progeny of three clones low in 6H-DMD values, df equals 28.

[e] Neutral detergent fiber.

[f] Acid detergent fiber.

[g] Acid detergent lignin.

*Significant P < .05; **significant P < .01.

The progeny of the three clones selected for high 6H-DMD values were actually 10.26 percent higher in leaves, 5.87 percent lower in total cell-wall quantity, 2.54 percent lower in hemicellulose, 2.50 percent lower in cellulose, and 0.84 percent lower in lignin than the progeny of the three clones selected for low 6H-DMD values. One significant composition change was found in the cell-walls. The progeny from the clones high in 6H-DMD had 3.61 actual percent less cell-wall hemicellulose. Calculated cell-wall cellulose and lignin were higher for progeny from clones high in 6H-DMD but the differences were not significant. As a result the ADL/ADF ratios were almost identical.

Detailed analysis of progeny from two clone combinations.--A detailed analysis of progeny from two clones high in 6H-DMD, H $\frac{B}{G}$, and progeny from two clones low in 6H-DMD, L $\frac{V}{D}$, was conducted. These two crosses were chosen because of their extremely different NDF mean values. Six hour *in vitro* dry matter disappearance analysis was performed on the progeny of each cross. Means and mean differences for all variables measured on these progeny are presented in Table 13. The same pattern of significant mean differences was found in these two clone combinations as in the three clone combinations. Greater quantity and composition differences were expressed between cell-wall means of these two clone combinations than between cell-wall

Table 13.--Mean values and mean differences between variables evaluated in progeny from three clones high and three clones low in 6H-DMD, and two clones high and two clones low in 6H-DMD. All values are expressed in percent except yield values which are expressed in grams.

	Three Clone Combinations			Two Clone Combinations		
	High[a] (1)	Low[b] (2)	Diff.[c]	High[d] (3)	Low[e] (4)	Diff.[f]
Yield	138.07	106.86	+31.21*	157.80	119.90	+37.90*
Leaves	45.46	35.20	+10.26**	44.49	30.70	+13.79**
NDF	36.02	41.89	-5.87**	36.18	44.34	-8.16**
ADF	30.48	33.81	-3.33**	30.15	34.68	-4.53**
ADL (Lignin)	6.98	7.82	-0.84**	6.75	7.61	-0.86*
Hemicellulose[g]	5.54	8.08	-2.54**	6.02	9.66	-3.64**
Cellulose[h]	23.50	26.00	-2.50**	23.40	27.08	-3.68**
Lig. of NDF[i]	19.42	18.74	+0.68	18.66	17.14	+1.52
Hemi. of NDF[j]	15.38	18.99	-3.61*	16.70	21.76	-5.06*
Cell. of NDF[k]	65.20	62.27	+2.93	64.64	61.10	+3.54
ADL/ADF	22.90	23.10	-0.20	22.39	21.94	+0.45
Protein	--	--	--	22.90	19.01	+3.89**
6H-DMD	--	--	--	43.05	37.37	+5.68**

[a] Three clones high in 6H-DMD, G23-12, B75, D20/D220-2.

[b] Three clones low in 6H-DMD, V232/V219-2, B9, D69/D52-9.

[c] Mean of combination (1) minus (2).

[d] Two clones high in 6H-DMD, G23-12, B75.

[e] Two clones low in 6H-DMD, V232/V219-2, D69/D52-9.

[f] Mean of combination (3) minus (4).

[g] Neutral detergent fiber minus acid detergent fiber.

[h] Acid detergent fiber minus acid detergent lignin.

[i] Acid detergent lignin divided by neutral detergent fiber.

[j] Hemicellulose divided by neutral detergent fiber.

[k] Cellulose divided by neutral detergent fiber.

*Significant $P < .05$; **significant $P < .01$.

means of the three clone combinations. In addition, protein content and 6H-DMD values were significantly different.

Correlations between the cell-wall components of the leaves and stems, and the cell-wall components obtained with the whole plant, are presented in Table 14. Neutral detergent fiber, ADF, and ADL content of leaves was not significantly correlated with the whole plant NDF, ADF, or 6H-DMD values. In contrast, stem NDF and ADF content was significantly and highly correlated with values for the whole plant. Correlation coefficients were not calculated within each family because of the small number of progeny (ten) and low level of variability.

Leaf and stem cell-wall analysis is presented in Table 15. A problem was encountered in analysis technique. Neutral detergent fiber extraction was made of leaf and stem samples. Since the quantity of sample was limited, the residue was then extracted with ADF solution. This was followed by extraction with ADL solution. When leaf and stem NDF contents were multiplied by their respective whole plant fractions, values were obtained that were similar to those of the actual whole plant. However, when leaf and stem ADF and ADL calculations were made to obtain estimates of whole plant values, discrepancies occurred. Apparently the ADF and ADL extractions of a NDF residue

Table 14.--Correlation coefficients among variables measured for whole plants and leaf and stem cell-wall components for combined progeny of two clones high and two clones low in 6H-DMD.

	Leaf NDF[a]	Leaf ADF[b]	Leaf ADL[c]	Stem NDF	Stem ADF	Stem ADL
Plant Yield	+.08[d]	+.05	+.17	-.30	-.26	+.10
Plant NDF	-.07	+.15	+.16	+.81**	+.76**	+.37
Plant ADF	-.05	+.22	+.16	+.70**	+.70**	+.50*
Plant ADL	+.09	+.18	+.33	+.54*	+.58**	+.57**
Plant 6H-DMD	+.11	-.22	-.04	-.85**	-.87**	-.47*

[a] Neutral detergent fiber.

[b] Acid detergent fiber.

[c] Acid detergent lignin.

[d] All correlations, 18 df.

*Significant $P < .05$; **significant $P < .01$.

Table 15.--Mean values and mean differences of cell-wall components measured for leaves and stems of progeny from two clones high and two clones low in 6H-DMD. All values are expressed in percent.

Variable	High[a]	Low[b]	Difference[c]
Leaf NDF[d]	16.50	15.87	+.69
Leaf ADF[e]	11.34	11.73	-.39
Leaf ADL[f]	2.14	2.17	-.03
Stem NDF	48.92	56.73	-7.81**
Stem ADF	35.73	41.56	-5.83**
Stem ADL	6.73	7.37	-.65
Leaf and Stem NDF[g]	34.50	44.18	-9.68**
Leaf and Stem ADF[h]	24.88	32.40	-7.50**
Leaf and Stem ADL[i]	4.68	5.79	-1.11**

[a] Progeny from the $H \frac{B}{G}$ combination.

[b] Progeny from the $L \frac{V}{D}$ combination.

[c] Actual difference between mean values of combinations.

[d] Neutral detergent fiber.

[e] Acid detergent fiber.

[f] Acid detergent lignin.

[g], [h], [i] Percent leaves times the corresponding cell-wall component value plus percent stems times the corresponding cell-wall component value.

*Significant $P < .05$; **significant $P < .01$.

give lower values than similar extractions on the original sample. The chemical implications are beyond the scope of this thesis. Due to these inconsistencies, all cell-wall components and cell-wall composition calculations of leaves and stems are omitted.

Of the actual 8.16 percent difference in NDF values for the whole plant between these two crosses, 2.1 percent to 2.5 percent was due to differences in percent leaves and the remaining 6.1 percent to 5.7 percent was due to differences in stem cell-wall quantity.

In order to obtain visual evidence that differences in chemical determination of cell-walls were directly related to actual cell-wall differences, a single stem cross section was made from five representative plants in each cross, Figures 2 and 3. A number of morphological similarities and differences were apparent. Dark and light differences among these cross sections were believed due to staining technique rather than differences in cell-wall composition. Stem shape was more nearly square on the progeny of the $H \frac{B}{G}$ cross, while that of the $L \frac{V}{D}$ cross stems appear more round. The thickness of the epidermis and outer cortex were nearly the same for all stem sections. Bundle caps and phloem tissues were of the same relative size and arrangement. The first major difference was the number of vascular bundles in each stem. An average of

Figure 2.--Cross section of a representative stem from five progeny of combination $H \frac{B}{G}$. Sections were taken from the middle of an internode five inches above soil level. 21 X. See Appendix, Table 23 for data.

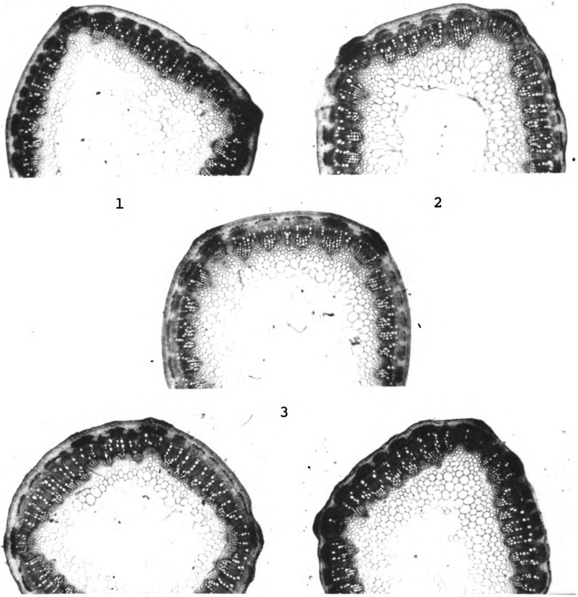
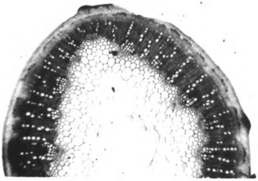
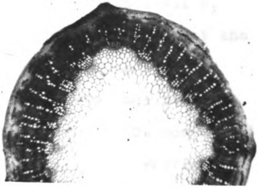


Figure 2

Figure 3.--Cross section of a representative stem from five progeny of the combination L $\frac{V}{D}$. Sections were taken five inches above soil level. 21 X. See Appendix, Table 23 for data.



1



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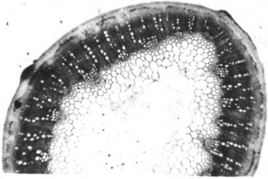

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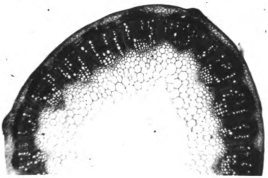

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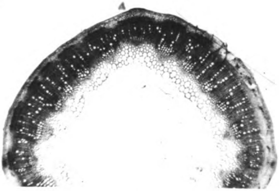


5

Figure 3

24 bundles was counted in the stems of L $\frac{V}{D}$, and 31 in the stems of H $\frac{B}{G}$ ($P < .01$).

The second major difference was in the radii of the xylem cells. The average number of radii cells of the xylem was significantly different, 22.2 versus 16.7 ($P < .01$). Xylem cell counts in a circumference revealed an average of 777 cells in the stems high in NDF content and 800 in the stems low in NDF content. No significant difference was expressed in xylem circumference cell numbers. To obtain estimates of total cell numbers, average radius cell numbers was multiplied by the average circumference cell numbers. Approximately 17,317 xylem cells were present in the stems high in NDF content compared to 13,335 in the stems low in NDF content. This represents a difference in estimated total xylem cell numbers in stems of 22.9 percent, five inches from the soil level. The raw data can be found in the Appendix, Table 23.

Changes in Cell-Wall Constituents During Ontogeny

Four *M. falcata* clones were vegetated so that seven cuttings at ten day intervals of the same clone were made over a two month period. Plants were separated into leaves and stems to compare cell-wall component changes in these two plant parts.

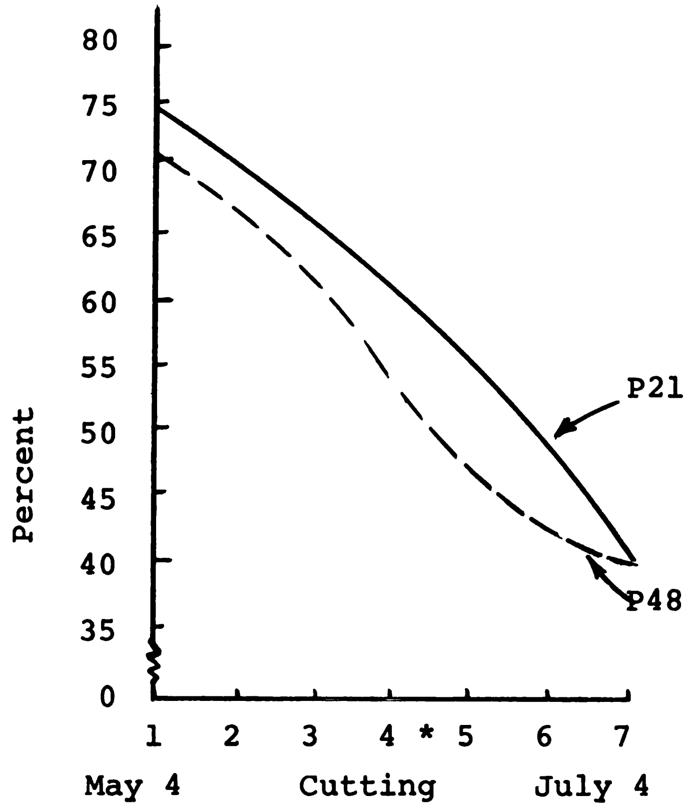
Analysis of variance of NDF, ADF, and ADL content is presented in Appendix, Table 24. Main effects and interactions were highly significant except genotype x cutting interactions. Average values for the four whole plants at each cutting are in Appendix, Table 25. The results are in general agreement with those reported by Terry and Tilley (1964), and Barnes and Ferrer (1968).

Detailed results obtained for two clones are presented in Figures 4 to 13. The reduction in percent leaves for both plants during ontogeny is shown in Figure 4. Seventy to 74 percent of the total plant sample consisted of leaves at first cutting and decreased to 38-40 percent in the final cutting. This decrease in percent leaves is similar to that reported by Terry and Tilley (1964). Plant P48 had fewer leaves throughout the growing period than plant P21. An actual 8 percent difference in leaves occurred at first bloom stage. Plant P48 had lower NDF, ADF, and ADL content than P21 throughout the growing period; however, the two plants became more alike in cell-wall components at late maturity, Figure 5. At the first bloom stage P48 was actually 4.34 percent lower in NDF, 2.89 percent lower in ADF, and .38 percent lower in ADL than P21.

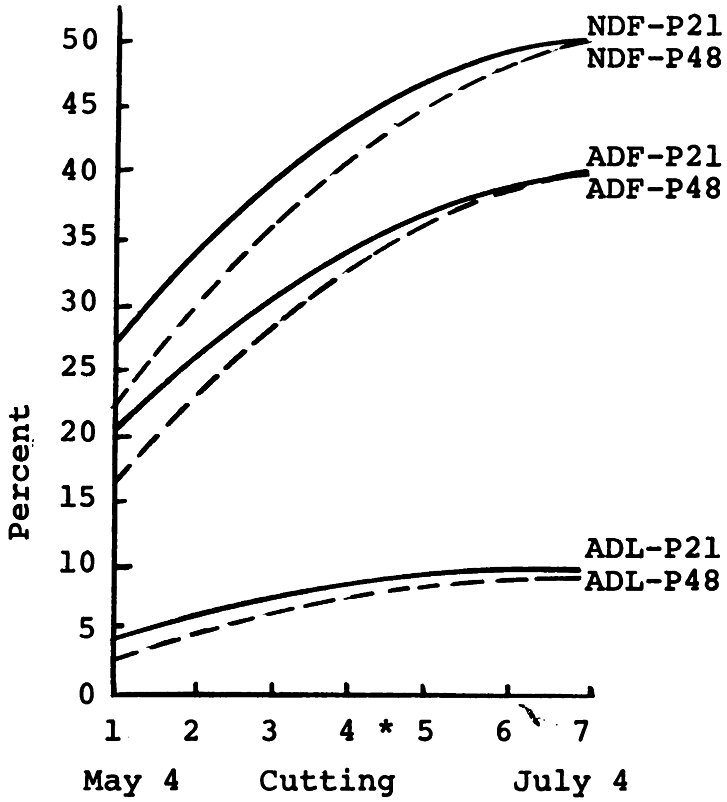
Changes in cell-wall components are shown in Figures 6 and 7. Lignin and hemicellulose increased at

Figure 4.--Changes in percent leaves of plant P21 and P48 during ontogeny.

Figure 5.--Changes in percent neutral detergent fiber (NDF), acid detergent fiber (ADF), and acid detergent lignin (ADL) of plant P21 and P48 during ontogeny.



Plant 21 and 48



Plant 21 and 48

*Approximate cutting date for first bloom.

Figure 4

Figure 5

Figures 6-7.--Changes in cellulose (C), hemicellulose (H), and lignin (L) of P21 and P48 during ontogeny. Cell-wall components expressed as percent of whole plant.

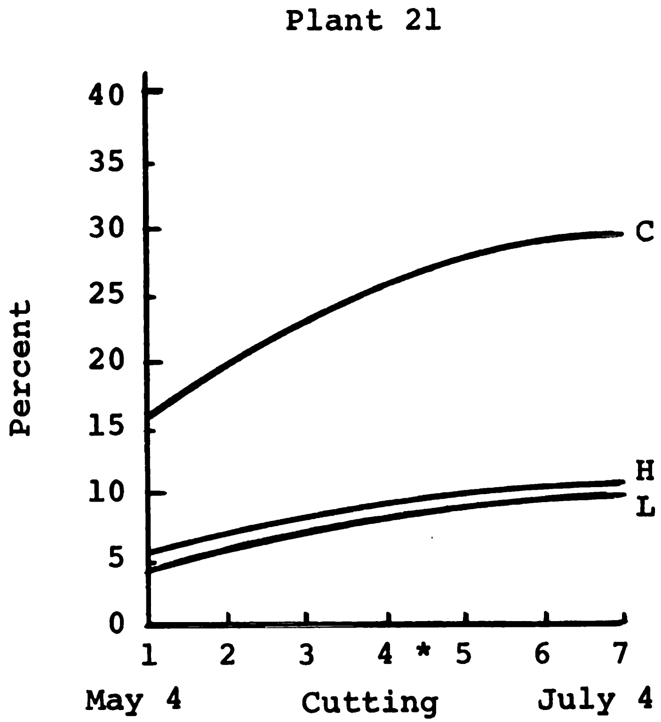


Figure 6

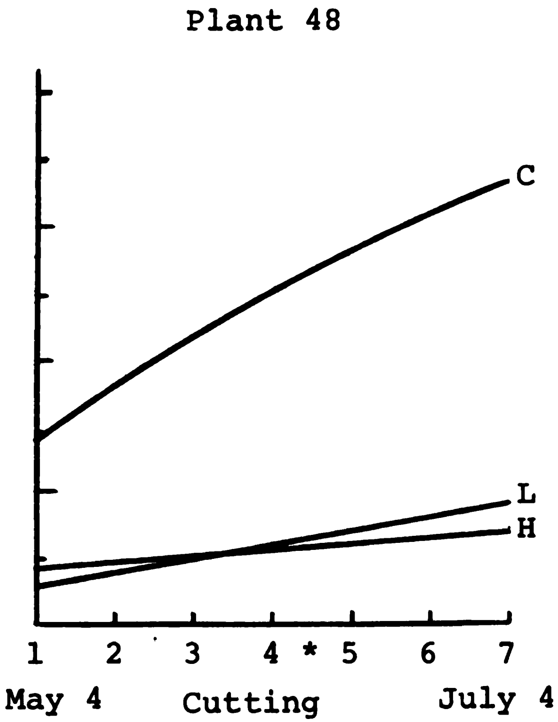


Figure 7

*Approximate cutting date for first bloom.

similar rates in P21. Cellulose and hemicellulose content nearly doubled while lignin increased slightly more than 50 percent during the growing period. An interaction occurred in hemicellulose and lignin content in P48. Lignin and cellulose were produced at a more rapid rate than hemicellulose. Cellulose and lignin more than doubled while hemicellulose increased less than 50 percent.

Changes in cell-wall components, expressed as percent of the cell-wall, for these two genotypes are presented in Figures 8 and 9. Cell-wall extracts of P21 and P48 contained an average of 59 percent and 64 percent cellulose, respectively, throughout all cuttings. Plant P21 had a higher quantity of hemicellulose and lignin in the cell-wall at each cutting than P48. The interaction of percent lignin and hemicellulose was again evident in P48.

Changes in cell-wall components of leaves and stems during ontogeny are presented in Figures 10 and 11. Cellulose, hemicellulose, and lignin contents were higher in the stems of P21 and P48 than the leaves. Stems of P21 increased from 23.1 to 38.4 percent in cellulose, 8.6 to 13.7 percent in hemicellulose and 5.7 to 13.0 percent in lignin. In the leaves, cellulose increased from 13.9 to 16.0 percent, hemicellulose from 5.7 to 6.9 percent, and lignin from 4.5 to 5.1 percent. The leaves and

Figures 8-9.--Changes in cellulose (C), hemicellulose (H), and lignin (L) of P21 and P48 during ontogeny. Cell-wall components expressed as percent of cell-wall.

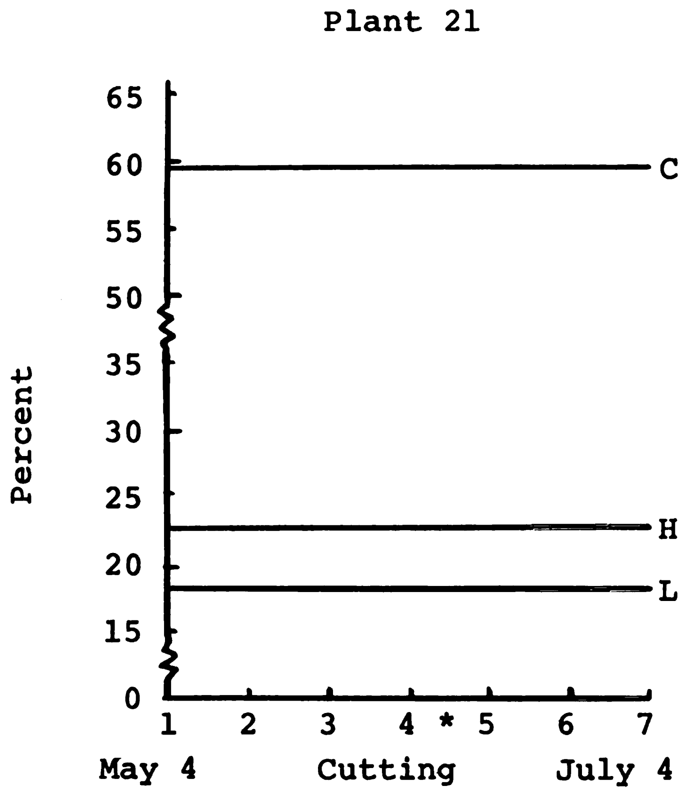


Figure 8

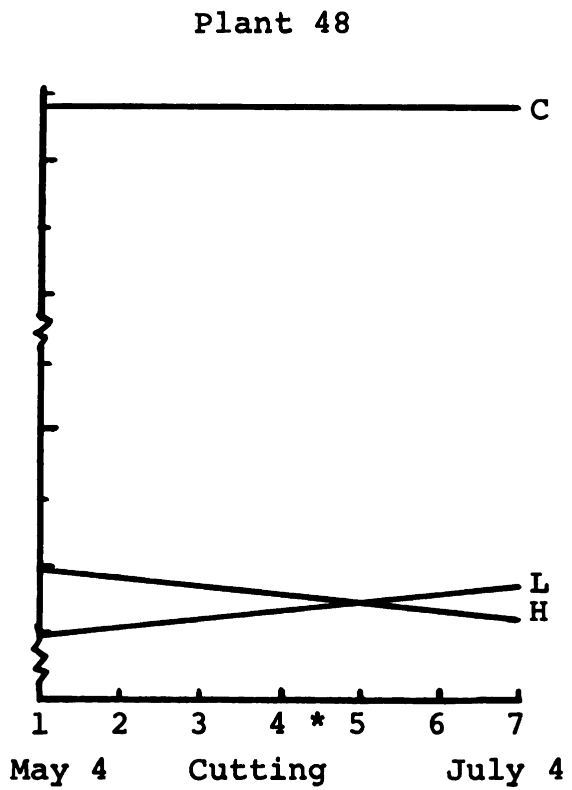
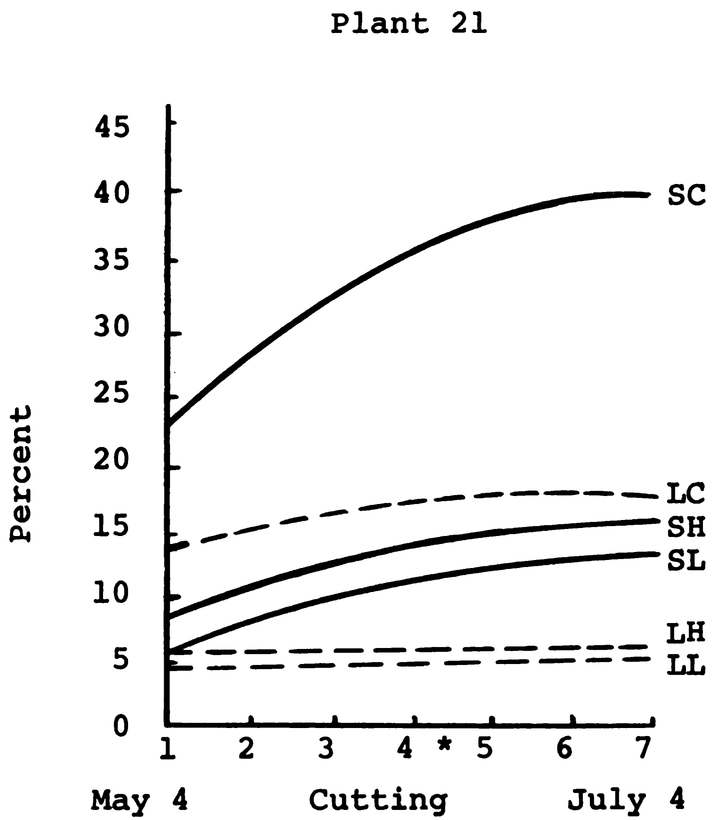
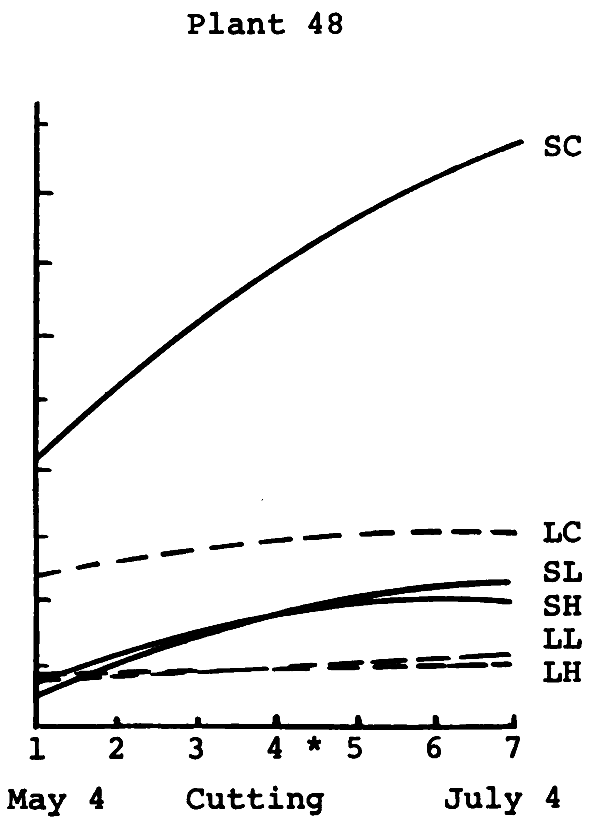


Figure 9

*Approximate cutting date for first bloom.

Figures 10-11.--Changes in cellulose, hemicellulose, and lignin content of leaves and stems of P21 and P48 during ontogeny. Cell-wall components expressed as percent of whole plant. (SC) stem cellulose, (LC) leaf cellulose, (SH) stem hemicellulose, (LH) leaf hemicellulose, (SL) stem lignin, (LL) leaf lignin.





*Approximate cutting date for first bloom.

Figure 10

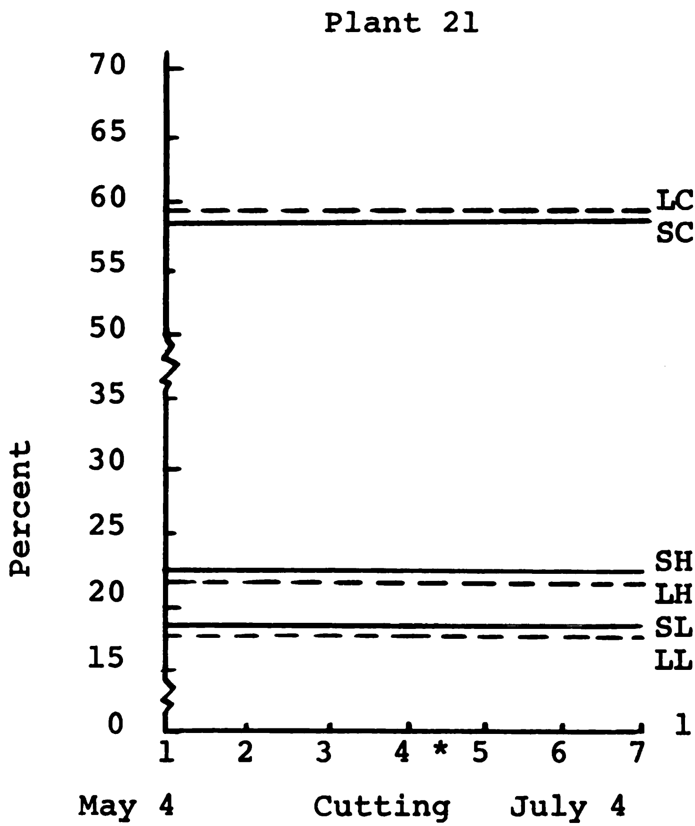
Figure 11

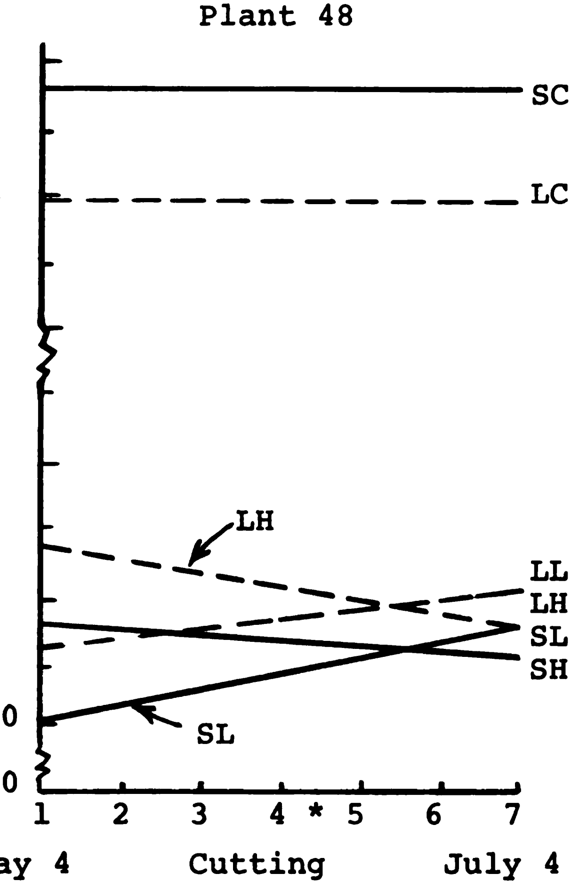
stems of P48 were all lower in quantity of cell-wall components than P21. Stem cellulose was an exception. P48 when mature contained a greater amount of stem cellulose than P21. The interaction of hemicellulose and lignin production occurred in both leaf and stem tissues of P48.

Changes in leaf and stem cell-wall components, expressed as percent of the cell-wall, are shown in Figures 12 and 13. No apparent differences existed between the percentage of leaf and stem cell-wall components from vegetative to late maturity in P21. On the average 59 percent of the leaf and stem cell-walls were cellulose, 22.5 percent hemicellulose, and 17.5 percent lignin. Major compositional differences occurred in P48. The cell-walls of the leaves had greater hemicellulose and lignin content and a significantly lower cellulose content than did the stems. The hemicellulose content of both leaf and stem cell-walls decreased as lignin content increased during ontogeny. Leaf and stem cellulose remained constant throughout the maturation process.

The summative equation of Van Soest (1967) was used to estimate digestibility of the _M_. _falcata_ forages. Table 16, contains the calculated true and apparent digestibility of first and final harvests. The ADL/ADF ratios from the chemical analyses of alfalfa by Barnes and Ferrer (1968) were substituted for the actual _M_. _falcata_ ratios

Figures 12-13.--Changes in cellulose, hemicellulose, and lignin content of leaves and stems of P21 and P48 during ontogeny. Cell-wall components expressed as percent of cell-wall. (SC) stem cellulose, (LC) leaf cellulose, (SH) stem hemicellulose, (LH) leaf hemicellulose, (SL) stem lignin, (LL) leaf lignin.





*Approximate cutting date for first bloom.

Figure 12

Figure 13

Table 16.--Application of Van Soest's summative system of calculating digestibility to first and final harvest of *M. falcata* clones.[a] Values expressed as percent.

	First Harvest May 4				Final Harvest July 4			
Component	Quant.[b]	$\frac{\text{ADL}}{\text{ADF}}$	Factor[c]	Dig.	Quant.[b]	$\frac{\text{ADL}}{\text{ADF}}$	Factor[c]	Dig.
1. Calculated digestibility for average first and final harvest of all four *M. falcata* clones.								
Cellular contents	76.15	--	98.0	74.63	50.37	--	98.0	49.36
Cell-wall constituents	23.85	19.21	45.8	10.92	49.63	21.92	41.5	20.60
Est. true dig.				85.55				69.96
Metab. fecal matter				-12.90				-12.90
Est. apparent dig.				72.65				57.06
2. Calculated digestibility for average first and final harvest of all four *M. falcata* clones using Barnes and Ferrer (1968) ADL/ADF ratio.								
Cellular contents	76.15	--	98.0	74.63	50.37	--	98.0	49.36
Cell-wall constituents	23.85	24.40	37.9	9.04	49.63	26.40	34.9	17.32
Est. true dig.				83.67				66.68
Metab. fecal matter				-12.90				-12.90
Est. apparent dig.				70.77				53.78
3. Calculated digestibility for first and final harvest of plant P21.								
Cellular contents	72.51	--	98.0	71.06	49.80	--	98.0	48.80
Cell-wall constituents	27.49	22.68	40.5	11.13	50.20	25.07	37.0	18.57
Est. true dig.				82.19				67.37
Metab. fecal matter				-12.90				-12.90
Est. apparent dig.				69.29				54.47
4. Calculated digestibility for first and final harvest of plant P48.								
Cellular contents	78.14	--	98.0	76.58	49.92	--	98.0	48.92
Cell-wall constituents	21.86	18.73	46.6	10.14	50.08	21.16	43.1	21.58
Est. true dig.				86.77				70.50
Metab. fecal matter				-12.90				-12.90
Est. apparent dig.				73.87				57.60

---

[a] Van Soest, J. P., and R. H. Wine. 1967. Acid-detergent fiber determinations of lignin, cellulose, and insoluble ash (silica) and their application to the estimation of digestibility in the summative equation. Paper presented at meeting of American Society of Animal Science, Reno, Nevada.

[b] Quantity equals quantity of cellular contents or cellular constituents.

[c] Percent of component digested.

in the second set of calculations. True and apparent digestibilities were also calculated for P21 and P48.

Weanling Vole Experiments with Selected Alfalfa Composites

Weanling meadow voles were used to determine if chemical components in forage affecting *in vitro* dry matter disappearance would alter growth, intake, or digestibility.

Experiment 1. Forage 6H-DMD values and weanling weight gain comparisons

Eight forages ranging from 28.8 percent to 39.1 percent in 6H-DMD values were fed *ad libitum* to weanlings for six days. The correlation between forage 6H-DMD values and vole weight gain was .78 ($P < .01$, 28 df). Some information was obtained on intake; however, problems of diet waste and variation in moisture content prevented valid conclusions. Modifications in alfalfa diet formulation were undertaken to reduce waste.

Experiment 2. Improvement in feeding procedures (waste reduction)

Seven adhesive substances were tested in alfalfa wafers. The texture of each wafer was carefully examined for adhesive and cohesive properties. Kelcoloid LV and cellulose gum retarded crumbling and diet breaking better than the other adhesives. These substances were incorporated into semi-synthetic diets at 2 percent and 5 percent for growth and feeding comparisons. No reduction in growth occurred at 2 percent with either adhesive but due to the hardness of the diets containing 5 percent of these ingredients, growth was below normal. Waste was reduced 66 percent and 78 percent at the 2 percent level and 78 percent and 90 percent at the 5 percent level for Kelcoloid LV and cellulose gum, respectively. Cellulose gum was tested in alfalfa feeding trials and gave a 63 percent reduction in waste.

Experiment 3. Vole weight gain, intake, and digestion of six selected forage composites

Chemical analysis of the six forage composites are listed in Table 17. The selected composite samples high in 6H-DMD were higher in percent protein, and ash, and lower in percent NDF than the low composites. The analysis of variance for gain/day, intake/day, apparent dry matter digestibility, and NDF digestibility is presented in the Appendix, Table 26, with mean values in Table 18.

Daily gain of voles was significantly affected by forages selected as high or low in 6H-DMD values, as well as dietary alfalfa levels. Daily gain was higher on all forages with high nutritive values at both 83 percent and 48 percent alfalfa. Part of the reason for the significant

Table 17.--Chemical analysis of six selected forage compositions fed to weanling voles. Values expressed as percent.

Composite	Protein	NDF[c]	Ash
Vernal High[a]	21.31	42.14	8.80
Vernal Low[b]	19.59	43.93	8.07
DuPuits High[a]	20.83	40.89	10.85
DuPuits Low[b]	17.26	45.68	9.25
MSB-C4 High[a]	20.35	40.41	9.31
MSA-C4 Low[b]	19.17	46.90	8.26

[a] High refers to clone composites with high 6H-DMD values.

[b] Low refers to clone composites with low 6H-DMD values.

[c] Neutral detergent fiber.

interaction terms in daily gain and intake was due to weight loss of voles fed the Vernal composite low in 6H-DMD, at 83 percent alfalfa.

No significant differences were detected in intake among forages and dietary alfalfa levels. All diets containing 83 percent alfalfa had lower apparent digestibility of the dry matter than the same forages fed at 48 percent alfalfa, but no differences were noted in apparent digestibility of the dry matter among forages high or low in 6H-DMD values. Digestion of the NDF fraction was

Table 18.--Mean response of weanling voles to six selected alfalfa forages differing in 6H-DMD values.

Forage	Gain/Day (g)	Intake/Day (g)	App. Dig.[c] (%)	NDF Dig.[d] (%)
Forage Fed at 83 Percent of Diet				
Vernal High[a]	.64	4.40	.52	.20
Vernal Low[a]	-.11	3.15	.46	.15
DuPuits High[b]	.79	4.46	.55	.20
DuPuits Low[b]	.21	4.09	.50	.21
MSB-C4 High[a]	.89	4.38	.53	.18
MSA-C4 Low[a]	.53	4.38	.54	.28
Forage Fed at 48 Percent of Diet				
Vernal High[a]	.89	4.02	.67	.09
Vernal Low[a]	.78	4.42	.73	.17
DuPuits High[b]	1.00	4.63	.73	.16
DuPuits Low[b]	.75	4.02	.70	.17
MSB-C4 High[a]	.89	3.59	.68	.02
MSA-C4 Low[a]	.72	3.67	.68	.14

[a] Mean of three voles.

[b] Mean of two voles.

[c] Apparent digestibility of dry matter equals intake minus feces production divided by intake.

[d] Neutral detergent fiber digestibility equals fiber intake minus fiber in feces divided by fiber intake.

significantly higher on all forages fed at 83 percent of the diet, and a trend toward greater NDF digestion was found on forages with low 6H-DMD and high NDF values.

DISCUSSION

Two Cycles of Selection for the 6H-DMD Character

The first cycle of selection for the 6H-DMD character was effective in both Vernal and DuPuits varieties. Although the character was believed to be quantitative, mass selection has shifted progeny means. General combining ability was significant within a cutting of the Vernal clones high in 6H-DMD. This result is in general agreement with those of Gil *et al*. (1967), Knight and Yates (1968), and Carlson *et al*. (1969). In unselected populations, a genetic basis for 6H-DMD differences among individual clones also exists. Environmental changes over different cutting dates resulted in many inconsistent clone and progeny responses.

A number of general relationships between 6H-DMD and clone yield occurred in each population. (1) All clone 6H-DMD values increased from first to second cutting and yield values decreased. (2) Cutting x clone interactions existed in most populations for 6H-DMD and yield. (3) Low 6H-DMD values were curvilinearly correlated with high yield in the first cutting of each population and linearly correlated in the second cutting. These relationships existed in varying degrees among populations.

The four DuPuits crosses were chosen as an example of how closely yield and 6H-DMD values are related in some genotypes. When growth and yield were depressed by environmental moisture stress, results were obtained similar to those reported by Gifford and Jensen (1967) and Kramer (1959). Clones with the greatest yield potential under near optimum first cutting environment, probably made the greatest changes in structure and cell-wall production under second cutting moisture stress. These changes may have been responsible for the drastic alteration in *in vitro* dry matter disappearance in the second cutting values.

The statement by Elliott (1958) about the vegetative buffering capacity of perennial species exposed to different environments, is applicable to these results. Further evidence of this genotype-environmental interaction and its implications for plant breeders have been expressed by Knight and Yates (1968) and Shenk (1967). These differences in *in vitro* digestibility among clones in the same environment and changes in different environments are believed closely related to changes in the structural composition of the vegetative tissue. When "yield" is substituted for *in vitro* digestibility in the last sentence the statement may also be correct.

Cell-wall production has been shown to be related to both digestibility and yield by the cell-wall analysis of single clones with high or low in 6H-DMD values. *In vitro* digestion values of these selected genotypes were more closely related to the cell-wall content of the plants than yield. Even though correlation coefficients were negative between *in vitro* digestibility and yield and positive between *in vitro* digestibility and cell-wall content, neither were significant. From the size of these two latter correlations alone, yield reduction is not a requirement for high digestibility in all genotypes.

Line breeding was utilized as a breeding method to select clones from the Vernal and DuPuits populations for the second cycle. This was done in an attempt to obtain some fixation of the 6H-DMD character without extensive inbreeding. Superior clones exhibiting stable *in vitro* values over cuttings from the previously unselected populations were included in the second cycle recombination.

The general and specific combining abilities of second cycle clones were significant in both high and low 6H-DMD combinations for leaf and NDF content as well as yield, with one exception. These results are similar to the findings of Gil *et al*. (1967), Knight and Yates (1968), and Carlson *et al*. (1969). The statistics imply that not all parental clones were equally effective in transmitting these traits; however, this initial analysis of all crosses not only helped identify the clones with the highest general combining effects, but also served as a basis for identifying unique two and three clone combinations.

An estimate of the progress in separating these progeny high and low in 6H-DMD can be obtained by comparing the progeny means of the first and second cycle. The differences between two clone combinations with the highest and lowest 6H-DMD mean in the DuPuits selection of the first cycle were 5.2 percent and Vernal 4.9 percent. In the second cycle the difference between the only two clone combinations evaluated for 6H-DMD was 5.7 percent. The second cycle of selection was responsible for a 9.6-16.3 percent improvement in separating these progeny means.

The first clue as to what effect 6H-DMD selection was having on plant composition came from evaluating all progeny for leaves and NDF content. A significant factor contributing to the difference in cell-wall quantity between the progeny from parental clones high and low in 6H-DMD values was the leaf content. Development of alfalfa varieties with high leaf content was suggested by Terry and Tilley (1964), and Mowat *et al*. (1965) to increase digestibility by reducing total cell-wall constituents.

In the specific comparison between the $H \frac{B}{G}$ and $L \frac{V}{D}$ crosses, percent leaves provided slightly more than 1/3 of the cell-wall differences. The remaining difference was due to stem cell-walls.

The magnitude of the 6H-DMD difference between the progeny of these crosses must have been related to differences in both leaf percentages and stem cell-wall content. The reduction in stem cell-walls was postulated to be important in breeding for greater digestibility by Macdonald (1946), Reid _et_ _al_. (1959), and Terry and Tilley (1964). The greater percent protein of the $H \frac{B}{G}$ cross was probably a function of the greater leaf content but protein was not believed a major factor in its high digestibility.

The difference in stem xylem cells between cross $H \frac{B}{G}$ and $L \frac{V}{D}$ was shown by histological sections to be due to changes in tissue proportions. The NDF analysis of the stems apparently estimated the changes in quantity of lignified xylem tissue to the relatively thin walled parenchyma and pith tissue. It can be assumed that these changes in tissue proportion were the result of cambial activity. The greater number of vascular bundles in the stems of the progeny from the $H \frac{B}{G}$ cross is believed related to their greater percent of leaves.

These differences in stem tissue proportions occurred in conjunction with differences in stem length and leaf content. Not only were the stems of the $L \frac{V}{D}$ cross longer than the $H \frac{B}{G}$ cross but the average stem length of all progeny from clones low in 6H-DMD were longer than progeny from clones high in 6H-DMD. Terry and Tilley (1964) reported that long stems were higher in structural carbohydrates than short stems. Since the proportion of xylem tissue in the leaf is lower than in stems, plants with short stems and a high percentage of leaves would have low NDF values. If in addition these shorter stems had smaller proportions of xylem tissue as shown in the $H \frac{B}{G}$, $L \frac{V}{D}$ comparison, further reduction in whole plant NDF totals would be expected. Stem elongation and cambial activity were shown to be hormone controlled (Digby and Wareing, 1966; and Wareing *et al.*, 1964) and may play an important role in dictating plant composition and digestion.

The only significant change in cell-wall composition in both the two and three clone combinations was hemicellulose content. The reason hemicellulose and not lignin changed in the cell-wall may be that lignification occurred after the cellulose microfibrils were laid down (Freudenberg, 1965). If lignification was a function of the quantity of cellulose in the cell-wall (Goodman and Siegel, 1959), and hemicellulose incorporation occurred prior to lignification (Clowes and Juniper, 1968), the deposition of hemicellulose may be independent of lignin encrustation of the cellulose matrix. This would make hemicellulose the most likely cell-wall component to respond to selection. Sullivan (1969) has stated that all preparations of lignin, hemicellulose, and cellulose are impure and contaminated with one another. The validity of this composition change in hemicellulose rests upon the chemical procedures and calculations used to detect it.

What influence, if any, a reduction in cell-wall hemicellulose has on nutritive value is open to question. If the ADL/ADF ratio actually dictates cell-wall digestibility (Van Soest, 1967) any reduction in cell-wall hemicellulose would have little influence. Actually, the ADL/ADF ratio appears to be rather constant in all alfalfa clones evaluated in these two cycles of selection. If the ADL/hemicellulose ratio is important (Sullivan, 1966), the cell-walls of the progeny from these two and three clone combinations high in 6H-DMD may be lower in digestibility.

The plant organ and related structural changes in the stems brought about by these two cycles of selection appear to have an affect on the morphology and growth habit of the plant. The progeny of the clones high in 6H-DMD are not as upright in growth habit as the progeny from clones low in 6H-DMD. These observations, combined with the knowledge that yield of space planted clones is a function of vertical and horizontal growth, help explain why greater mean yields were found for all progeny from clones high in 6H-DMD regardless of the positive yield-NDF relationship within populations.

Finally, concern has been expressed by Gil *et al.* (1967) and others that development of varieties high in digestibility may as a consequence reduce yields. This conclusion is drawn from the physical and statistical evidence; the opposite relationships must also be considered. Varieties developed for higher yield may as a consequence have low digestibility. Neither of these alternatives would be acceptable; however varieties high in digestibility have the potential of greater nutritional efficiency. This nutritional improvement expressed in terms of animal production may more than compensate for any reduction in yield.

Cell-Wall Quantity and Composition Changes During Ontogeny

There are at least three opportunities for the expression of gene differences controlling cell-wall production during growth. First, leaf cell differentiation and stem cell additions are the result of genes influencing primary meristems. Second, stem calls may be influenced by genes related to hormone controlled cambial activity in expanding xylem tissue (Digby and Wareing, 1966). Secondary cell-wall thickening occurs within both leaves and stems, providing a third opportunity for gene differences to be expressed. All three of these phenomena were believed responsible for the cell-wall differences among these *M. falcata* clones.

There was a striking difference between P21 and P48 in quantity of cell-walls. A genetic difference probably exists between these clones for the production of cell-walls. The increase in quantity of cell-walls in leaves was small relative to stems in either plant; therefore, the primary site of gene influence was either on cambial meristems or secondary cell-wall thickening processes of the stems.

Nevin *et al*. (1967) postulated that cell-wall composition is under genetic control. The genes influencing cell-wall composition in P21 produced similar percentages of cellulose, hemicellulose, and lignin throughout growth and differentiation processes even though cell-walls were differentiated by both primary and cambial meristems. Compositional differences in cell-walls existed between P21 and P48, as well as within the plant parts of P48. The apparent exchange of hemicellulose for lignin that occurred in both leaves and stems of P48 may be related to cell-wall thickening or maturing processes. Sullivan (1966) pointed out that differences in chemical bonding between hemicellulose and lignin as the plant approaches maturity may cause lower extraction yields of hemicellulose. This probably explains the lignin-hemicellulose interaction in P48.

The application of these chemical determinations to the concept of alfalfa digestibility was illustrated with Van Soest's summative equation. The calculated digestibilities of the forage from these *M. falcata* clones were slightly higher than *in vitro* digestibility values reported by Terry and Tilley (1964) at late maturity, but similar at the early vegetative stage. Although differences were apparent between the digestibility values calculated with actual ADL/ADF ratios and those of Barnes and Ferrer (1968), these digestibility values were remarkably similar considering that species, environment, and cutting dates were not the same in each experiment.

The cell-wall ADL/ADF ratio increased from 19.2 percent to 21.9 percent for all four genotypes during the maturation process. The cell-walls of the first cutting forage were estimated to be 45.8 percent digestible and the final cutting cell-walls 41.5 percent digestible: nevertheless, the microflora would digest nearly double the quantity of cell-walls in the mature forage.

Assuming these calculations representative of *in vivo* digestibility, the decrease in digestion of alfalfa forage harvested at different stages of maturity was influenced primarily by the increase in cell-wall quantity. This increase was primarily a phenomena of stem origin. Cell-wall compositional changes, including extent of lignification were of secondary importance.

In the specific case of the digestion of P21 versus P48, these general conclusions are valid in terms of the maturation processes; however, the low ADL/ADF ratio of P48 was unimportant in determining its calculated digestibility compared to P21 in the first harvest, but entirely responsible for the high calculated digestibility of P48 in the final harvest.

Weanling Vole Response to Selected Alfalfa Composites

The chemical components in alfalfa forages which affect *in vitro* dry matter disappearance appeared to be responsible for growth differences of weanling meadow voles. Only after cellulose gum was added to the diet formulation at 2 percent was waste reduced so that accurate intake values could be obtained. This improvement in feeding technique allowed more detailed study of the effects of specific chemical components in the forage on growth and digestion.

Regardless of the level of alfalfa in the diets of experiment 3, daily gain of voles was lower on the forages low in 6H-DMD and high in NDF. The reason for this response seems to be related to NDF digestion. In this experiment and in the results reported by Keys and Van Soest (1968), as the alfalfa content increased in the diet apparent digestibility of the dry matter decreased. With increasing dietary NDF content, digestibility of NDF increased.

Low protein was believed primarily responsible for the low weight gains of voles fed forages with low 6H-DMD values at 48 percent of the diet. The detrimental effects of high fiber content cannot be ruled out but sufficient energy for growth should have been supplied by the addition of 45 percent readily available carbohydrate to these diets. The general weight loss of all voles fed these forages at 83 percent of the diet was probably a function of the high fiber and low available energy for growth, since protein levels should have been adequate (Shenk and Elliott, 1968). Differences in weight gains among voles fed the forages high and low in 6H-DMD at 83 percent of the diet were believed to be a result of differences in the available energy of these forages. Factors other than fiber may have been responsible for the marked low responses of voles fed the low Vernal forage at 83 percent of the diet.

These vole studies were preliminary and conducted with small numbers of animals and forage samples. The weanling vole is not expected to replace the laboratory rumen *in vitro* or chemical procedures as mass screening techniques; however, at present no other assay has been developed that can provide the plant breeder with a better demonstration of animal growth potential of a few selected genotypes or breeder lines.

SUMMARY AND CONCLUSIONS

Two cycles of selection in alfalfa have been made in opposite directions for nutritive quality. Six hour *in vitro* and chemical cell-wall procedures were used to select parental clones and evaluate progeny. Changes in chemical composition and related characteristics of the progeny as a result of selection have been studied.

Asexually reproduced *M. falcata* clones were used to evaluate quantity and composition changes in leaf, stem, and whole plant cell-walls during ontogeny. Finally, weanling meadow voles were used to assess the nutritive quality of selected alfalfa genotypes. The following conclusions may be stated.

1. One cycle of mass selection and breeding of Vernal and DuPuits clones high or low in 6H-DMD was effective in developing progeny with significantly different 6H-DMD values.
2. Environmental differences from one cutting to the next caused the 6H-DMD values of many clones in the unselected populations to be variable.

3. Three general relationships were found between clonal dry weight yield and 6H-DMD values in the first cycle. (1) The yield of all clones decreased from first to second cutting and 6H-DMD values increased. (2) Significant cutting x clone interactions existed in most populations for both yield and 6H-DMD values. (3) All correlations between yield and 6H-DMD values in each cutting were either negative or near zero.
4. Chemical cell-wall analysis of clones selected for high or low 6H-DMD values from each population, revealed a close relationship between the quantity of cell-walls and the *in vitro* digestibility value of the clone.
5. General combining abilities of second cycle clones with both high and low 6H-DMD values were significant for yield as well as leaf and NDF content. All specific combining effects were significant with the exception of leaf content in the crosses between clones with low 6H-DMD values.
6. Complete cell-wall analysis of progeny from three clones with high and three clones with low 6H-DMD values selected from the second cycle had significant differences in average cellulose, hemicellulose, and lignin content. The only

significant composition change was lower hemicellulose content in cell-walls of the progeny from clones with high 6H-DMD values.

7. Progeny from two clone combinations chosen for their differences in NDF content were significantly different in protein content and 6H-DMD values. Leaf content was higher in progeny of the cross between clones with high 6H-DMD values than the progeny from the two clones low in 6H-DMD, but the NDF content of the leaves of these progeny groups was the same.
8. Stems of progeny from crosses between clones with high or low 6H-DMD values were significantly different in cell-wall content. Histological sections of the stems showed fewer vascular bundles and greater xylem cell numbers in the stems of progeny with low 6H-DMD values.
9. Quantity and composition changes in cell-wall constituents were expressed among four _M_. _falcata_ clones during ontogeny. Leaves were lower and remained relatively constant in cell-wall constituents compared to the stems.
10. The application of the summative equation of Van Soest to the _M_. _falcata_ data revealed that the decrease in digestibility of forage harvested at

different stages of maturity was influenced primarily by the increase in cell-wall quantity. Composition changes including degree of cell-wall lignification were of secondary importance.

11. A significant positive correlation existed between weanling vole daily gain and the 6H-DMD value of the forage: in addition, weanlings made significantly higher daily gains and digested smaller quantities of NDF on forages with low NDF values.

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APPENDIX

Table 19.--Individual clone analysis of five unselected alfalfa populations. Ranges, means, and standard deviations for 6H-DMD and yield.

Population and Harvest	No.[a]	6H-DMD (%)			Yield (g)		
		Mean	Range	SD[b]	Mean	Range	SD[b]
MSA-C4							
First Cut[c]	45	31.9	27.7-37.7	2.39	119.6	51-282	50.4
Second Cut[d]	45	41.3	35.5-47.9	2.84	57.3	30-124	27.2
MSB-C4							
First Cut[c]	68	36.6	31.0-42.5	2.77	120.0	32-306	48.5
Second Cut[d]	68	45.2	38.7-51.6	2.43	80.0	15-177	31.2
Vernal							
First Cut[c]	45	34.5	29.0-39.1	2.43	166.3	57-254	51.7
Second Cut[d]	45	41.5	35.4-48.6	2.82	67.7	27-118	27.0
Culver							
First Cut[c]	51	36.8	28.9-41.6	3.40	133.3	33-248	50.2
Second Cut[d]	51	40.4	35.3-44.7	2.38	84.0	43-119	17.6
Glutinosa							
First Cut[c]	43	36.0	30.1-41.9	2.54	121.8	14-259	52.9
Second Cut[d]	43	40.0	32.6-46.9	3.29	57.3	22-113	19.4

[a] Number of clones in each population.

[b] Standard deviation.

[c] Date of harvest June 10, 1967.

[d] Date of harvest July 30, 1967.

Table 20.--Mean squares for 6H-DMD and yield of five unselected populations.

		6H-DMD		Yield	
Populations	Clone No.	Cutting MS	Clone MS	Cutting MS	Clone MS
MSA-C4	45	2010.75**	8.75*	59341.34**	2415.89**
MSB-C4	68	2501.75**	8.77*	94343.56**	2338.39**
Vernal	45	715.53**	6.66	145829.40**	2022.31**
Culver	51	303.12**	7.80	61913.42**	1992.82**
Glutinosa	43	345.21**	10.69+	89477.63**	2270.13**

+Significant $P < .10$; *significant $P < .05$; **significant $P < .01$.

Table 21.--Mean squares for 6H-DMD and yield of four DuPuits families.

Source	df	MS 6H-DMD	MS Yield
Cuttings	1	543.95**	26912.00**
Crosses	3	33.42**	4814.96**
Cut x Crosses	3	19.63**	2274.59**
Clones Within Crosses	32	5.96	605.25*
Cut x Clones Within Crosses	32	3.75	274.73

*Significant P < .05; **significant P < .01.

Table 22.--Chemical and *in vitro* values of selected alfalfa clones. Values expressed as percent.

Clone	6H-DMD	TT[e]	NDF	ADF	ADL
DuPuits High[a]					
First Cut[c]	40.1	63.84	39.59	32.28	6.13
Second Cut[d]	44.1	71.02	29.59	21.67	3.69
DuPuits Low[b]					
First Cut[c]	32.1	49.28	47.05	36.73	6.01
Second Cut[d]	35.9	54.64	43.65	31.07	7.58
Vernal High[a]					
First Cut[c]	37.6	--	42.32	35.15	6.55
Second Cut[d]	42.1	--	39.44	30.37	6.95
Vernal Low[b]					
First Cut[c]	35.9	59.06	46.23	38.39	7.54
Second Cut[d]	36.9	59.19	45.88	37.78	8.60
MSA-C4 High[a]					
First Cut[c]	34.9	--	45.60	37.22	7.25
Second Cut[d]	41.4	--	37.27	29.75	5.96
MSA-C4 Low[b]					
First Cut[c]	31.3	--	47.42	38.10	8.65
Second Cut	38.8	--	37.82	28.17	6.08
MSB-C4 High[a]					
First Cut[c]	42.9	69.24	33.47	26.42	5.16
Second Cut[d]	42.6	66.84	35.98	27.57	6.32
MSB-C4 Low[b]					
First Cut[c]	33.7	63.61	41.76	35.28	7.21
Second Cut[d]	34.6	59.96	43.84	34.10	7.67
Glutinosa High[a]					
First Cut[c]	41.3	--	37.42	32.42	6.40
Second Cut[d]	39.1	--	36.81	29.00	6.61
Glutinosa Low[b]					
First Cut[c]	34.9	--	39.90	34.62	7.47
Second Cut[d]	33.5	--	41.47	32.57	6.86

[a] High refers to clone selected for high 6H-DMD values.

[b] Low refers to clone selected for low 6H-DMD values.

[c] Date of harvest June 10, 1967.

[d] Date of harvest July 30, 1967.

[e] Tilley and Terry *in vitro* procedure. Missing values due to limited forage.

Table 23.--Xylem cell counts of five stems, one from each of five H $\frac{B}{G}$ and five L $\frac{V}{D}$ progeny.

	Progeny H $\frac{B}{G}$					
	1	2	3	4	5	$\bar{X}^c$
Plant Number[a]						
Bundles[d]	39	26	31	31	26	31
Radii[e]	16.5	17.5	14.1	16.6	18.7	16.7
Circum.[f]	875	766	784	807	769	800
Total[g]	14,483	13,405	11,054	13,396	14,380	13,335

	Progeny L $\frac{V}{D}$					
	1	2	3	4	5	$\bar{X}^c$
Plant Number[b]						
Bundles[d]	25	26	25	22	22	24
Radii[e]	22.6	23.4	22.7	21.1	21.3	22.2
Circum.[f]	823	724	816	754	766	777
Total[g]	18,600	16,942	18,523	16,205	16,316	17,317

[a] Plant number page 57.

[b] Plant number page 59.

[c] Average cell numbers of each combination.

[d] Number of vascular bundles in each stem.

[e] Average xylem radii cells in each stem.

[f] Average number of xylem cells in a circumference.

[g] Estimated total cell numbers. Average xylem radii cells times the average circumference cell numbers.

Table 24.--Analysis of variance table of mean squares for four *M. falcata* clones.

Source	df	NDF[a]	ADF[b]	Hemi.[c]	Cell.[d]	ADL[e]	Hemi./NDF	Cell./NDF	ADL/NDF
Genotype	3	.007775**	.001834**	.002148**	.000355	.000822**	.003610**	.006103**	.001881**
Cutting	6	.050916**	.035302**	.001585**	.019235**	.002435**	.001076**	.000695	.001280**
Genotype X Cutting	18	.000452	.000363	.000059	.000281	.000032	.000348	.000504	.000260
Leaf-Stem	1	1.179170**	.792397**	.038310**	.523669**	.027728**	.005648*	.015735**	.002529**
Genotype X Leaf-Stem	3	.001552*	.000399	.001003**	.000559**	.000357**	.002785**	.007082**	.001159**
Cutting X Leaf-Stem	6	.023310**	.015849**	.000810**	.008659**	.001088**	.000445	.001120**	.000420*
Error	18	.000415	.000163	.000191	.000132	.000017	.000826	.000627	.000130

[a] Neutral detergent fiber.

[b] Acid detergent fiber.

[c] Hemicellulose equals neutral detergent fiber minus acid detergent fiber.

[d] Cellulose equals acid detergent fiber minus acid detergent lignin.

[e] Acid detergent lignin.

*Significant $P < .05$; **significant $P < .01$.

Table 25.--Average cell-wall composition of four *Falcata* clones harvested at seven stages of first growth maturity.

	Percent of Whole Plant			Cell-Wall Components as Percent of Cell-Wall		
Cut[a]	NDF[b]	ADF[c]	ADL[d]	Hemi.[e]	Lig.[f]	Cell.[g]
1.	23.85	18.69	3.62	21.50	15.06	63.45
2.	28.41	22.07	4.45	22.21	15.61	62.19
3.	32.53	25.59	5.13	21.32	15.71	62.97
4.	39.48	31.28	6.34	20.75	16.04	63.22
5.	44.11	35.19	7.34	20.17	16.65	63.18
6.	48.17	38.41	8.37	20.25	17.35	62.41
7.	49.63	40.70	8.90	17.81	18.02	64.17

[a] Cuttings made at ten day intervals between May 4 and July 4, 1968.

[b] Neutral detergent fiber.

[c] Acid detergent fiber.

[d] Acid detergent lignin.

[e] NDF minus ADF divided by NDF.

[f] ADL divided by NDF.

[g] ADF minus ADL divided by NDF.

Table 26.--Analysis of variance of weanling vole response to six selected alfalfa forages, differing in 6H-DMD values.

		Mean Squares			
Source[c]	df	Wt. Gain	Intake	App. Dig.[a]	NDF Dig.[b]
V	2	.1158**	.2614	.0016	.0039
$L_{(V)}$	5	.0323	.9577	.0006	.0072
S	1	1.1254**	.5735	.0013	.0177+
DA	1	1.0035**	.0841	.2549**	.0556**
V x S	2	.0307	.2219	.0012	.0088
DA x V	2	.1879**	1.1050**	.0019	.0105
DA x S	1	.2815**	.6834	.0018	.0068
DA x V x S	2	.0286	.7051	.0020	.0030
Error	15	.0144	.2542	.0016	.0043
C.V.		18.00%	12.36%	6.62%	3.74%

[a] Apparent digestibility of dry matter equals dry matter intake minus feces production divided by intake.

[b] Neutral detergent fiber digestibility equals fiber intake minus fiber in feces divided by fiber intake.

[c] V equals varieties, L equals litters, S equals 6H-DMD selections, DA equals dietary alfalfa (two levels), 48 percent and 83 percent. +Significant $P < .10$; *significant $P < .05$; **significant $P < .01$.