PARTITION, ANATOMY, CHEMISTRY AND DIGESTIBILITY OF ALFALFA AND BIRDSFOOT TREFOIL

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A DISSERTATION

Submitted to
Michigan State University
in partail fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Crop and Soil Scieces

1986

ABSTRACT

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Alfalfa (*Medicago sativa L.*) and birdsfoot trefoil (*Lotus corniculatus L.*) were grown in the greenhouse. The plants were collected at early bud, 10% bloom and full bloom. The morphological components of each legume were separated and several variables were evaluated so as to determine their level of changes along the stem and maturity and to compare their levels between both alfalfa and trefoil.

In chapter I, alfalfa had a higher leaf-stem ratio than trefoil, particularly in the middle layer, at blooming stages. Within both species, the leaf-stem ratio declined down the stem. The greatest concentration of both leaf and stem was found in the median portion of the shoot of both alfalfa and trefoil.

In chapter II, the percentage area of vascular bundles, sclerenchyma, xylem and phloem was greater in leaves of alfalfa and in trefoil. Mesophyll cells area was greater in trefoil than in alfalfa. The amount of epidermis, sclerenchyma and mesophyll cells were correlated with the relative feeding value (RFV).

The epidermis, bundle sheath, interfascicular xylem, vascular bundle and pith parenchyma cells of stem were affected by the location along the shoot. Several tissue types such as pith parenchyma, bundle sheath, epidermis, vascular bundles, xylem were correlated with stem IVDMD and RFV.

In chapter III, the trefoil leaf and stem had a higher level of NDF, ADF and wall protein than alfalfa. The levels of p-lignin and cellulose were higher in the leaf and stem of trefoil than in alfalfa. Cellular content in alfalfa was higher than in trefoil in both leaf and stem. Basal leaves contained greater NDF, ADF and cellulose than the upper and median leaves. In the stem, wall protein level was higher in the upper than in the two lower stem portions.

In chapter IV, leaf digestibility of alfalfa was higher than trefoil, particularly at the two upper layers. Stem digestibility declined from top to bottom in both alfalfa and trefoil.

In chapter V, digestion of most living tissue types in both leaf and stem was partial after 6 or 12 hours and showed visible degradation after 12 or 24 hours of fermentation. Digestion of leaf and stem of both species was characterized by the bacterial specificity, the surface potential of tissues and the interaction between bacteria and substratum.

ACKNOWLEDGEMENTS

The author expresses his deep gratitude to his parents for their great generosity and constant support. The author wants to extent his gratitude to Dr. Milo B. Tesar for making realisable the research work and the manuscript.

The author wants to thank the Department of Animal Science, the Department of Animal Husbandry and the Department of Botany and Plant Pathology and also the Department of Plant Science of Laval University to have allowed him to use their facilities to do the research work and the analysis of the data.

The appreciation of the author are extended to the members of his committee Dr. R. Emery, Dr. M. Yokoyama, Dr. C. Cress and Dr. S. Flegler and to the direction of Agriculture-Québec for their service and support.

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PREFACE

Alfalfa (*Medicago sativa L.*) and birdsfoot trefoil (*Lotus corniculatus L.*) are perennial legumes used primarily to supply forages to livestock. Alfalfa is considered by many to be the best forage crop although several factors such as soil fertility, acidity and moisture limit its use. Birdsfoot trefoil is a valuable alternative to alfalfa under some growth condition.

The high feeding value of both forage legumes is well known. However, controversial reports have been found about the difference of the nutritive value of the hay of the legume species as they matured. For instance, birdsfoot trefoil was found to retain more its digestibility than alfalfa with maturity but no explanation was given to support such an observation.

The main objective of the present study is to compare changes in feeding parameters between both alfalfa and trefoil with maturity. Nine experiments were conducted and distributed into five distinct chapters. Four chapters were related respectively to changes in partition of plant parts, histology, chemistry, and digestibility of both species with maturity. The fifth chapter is on ruminal bacteria activity in the degradation process of tissue types of both leaf and stem.

In section I, three different aspects of partition of leaf and stem studied: 1) on selected groups of morphological development collected at early bud, 10% bloom and full bloom of the whole plant, 2) along the shoot of plants harvested at various growth stages, and 3) as contri-

bution of each plant layers to the total amount of leaves and stems.

In chapter II, a quantitative evaluation of anatomical components was done in leaf and in stem of both alfalfa and birdsfoot trefoil, at three maturity stages and at different positions along the stem. The degree of association between the amount of various tissue types in leaf and in stem and the *in vitro* dry matter digestibility (IVDMD) and the relative feeding value (RFV) of leaf and stem was determined on all data.

Fibrous fractions and the cell wall constituents were determined in chapter III. In the first experiment, the feeding parameters were determined in ten groups of plant development of plants harvested at early bud and at full bloom within each species studied. In a second experiment, the same parameters were evaluated vertically and horizontally in leaf and in stem from a selected population of plants with 18 nodes. Changes in the concentration of the feeding parameters were obtained on both species at different positions on the shoot from early bud and full bloom plants.

In chapter IV, the digestibility of both alfalfa and trefoil forage was investigated in order to determine the degree of variation in digestibility among three groups of plants having varing development within the same stage of maturity. Another experiment was performed on alfalfa and trefoil to study the changes of the IVDMD of leaf and stem within a selected population of plants with 18 nodes.

In chapter V, the rate of degradation of the leaf and the stem tissue types from various position along the shoot was examined on a scanning electron microscope of both species collected at early bud, 10% bloom and full bloom. The association of the bacteria with the ultrastructure of the various tissue types and the interrelationships between the ruminal bacteria was determined.

CHAPTER I

MORPHOLOGICAL STUDY
OF
ALFALFA AND BIRDSFOOT TREFOIL

ABSTRACT

Alfalfa (*Medicago sativa L.*) and birdsfoot trefoil (*Lotus corniculatus L.*) were grown in the greenhouse to determine the partition of leaf and stem at early bud, 10% bloom and full bloom. Leaves and stems were studied: a) at the same maturity stage; b) in the top, middle and bottom of the plant; and c) in each of the three plant layers.

The partition of leaf and stem fractions on alfalfa was affected by the level of plant development. Alfalfa with low number of nodes(10 to 16) had a mean leaf-stem ratio lower than those with a higher node number. Trefoil leaf-stem ratio was greatly affected by the stage of maturity, declining from 0.68 at early bud to 0.47 at full bloom. From early bud to blooming stages, the leaf to stem ratio decreased for plants with 18, 20 to 22 nodes and increased for plants with lower numbers.

Alfalfa had a higher leaf-stem ratio at blooming stages than trefoil, particularly in the middle layer. The leaf-stem ratio declined markedly down the stem from 1.39 to 0.25 on alfalfa and from 1.26 to 0.19 on trefoil. The proportion of leaf to stem decreased from 0.67 at early bud to 0.43 at full bloom in trefoil and decreased from early bud (0.71) to 10% bloom (0.64) on alfalfa.

The leaf-stem ratio at the upper layer declined from early bud (2.07) to full bloom (0.80) on alfalfa and from 1.98 to 0.75 on trefoil. The ratios of the basal segment on both species were unchanged with maturity. At the median layer, trefoil had a lower leaf-stem ratio of 0.56 at full bloom compared to 0.72 at early bud and 0.69 at 10% bloom. Alfalfa was more leafy with a ratio of 0.80 at early bud, 0.84 at 10%

bloom and 0.96 at full bloom.

The greatest concentration of both leaf and stem was found in the median portion of the shoot of both alfalfa and trefoil. Trefoil had more uniform leaf distribution among the upper layers than alfalfa in the upper layer while alfalfa was more leafy in the median portion. Trefoil had a higher proportion of stem in the upper layer and a lower proportion in the bottom layer than alfalfa. The species were similar in the median segment.

INTRODUCTION

In animal feeding, leaves are consumed in greater quantity than stems (Larendo and Minson, 1973). Leaves have the highest concentrations of constituents important in nutrition (Sotola 1933, Mowat et al 1965, Smith 1969, Smith 1970, Martin 1970, Sullivan 1973). Consequently, forage quality would be greatly influenced by any morphological changes that increase the proportion of leaves.

Woodman and Evans (1935) found that leaf-stem ratio would be an important feature in the variations of some chemical components of legumes such as alfalfa. Since then, it has been well documented that the leaf-stem ratio would have an effect on nutritive value of legumes (MacDonald 1946, Reid et al 1959, and Ulyatt 1973). Variations of the leaf-stem ratio could be influenced by several factors such as species, maturity stages, plant development, and location on the stem.

Terry and Tilley (1964), Sullivan (1973), and Hoveland and Monson (1980) have studied the effect of maturity on the digestibility of alfalfa. All these authors reported a decrease in digestibility as the alfalfa matured; a reduction in leaf-stem ratio in maturing plants was partially responsible for their lower digestibility.

Lowe (1981) reported the possibility of using the leaf-stem ratio of alfalfa to select genotypes which would have higher digestibility but no further research was conducted. Finally, there is no published research a) to document the relationship that exists between the leaf-stem ratio and morphological development of legumes horizontally and vertically on the stem and b) to establish the contribution of each

morphological component: leaf and stem from various positions on the stem to the total leaves and total stems.

METHODOLOGY

Alfalfa (*Medicago sativa L.*) cultivar "Saranac" and birdsfoot trefoil (*Lotus corniculatus L.*) cultivar "Viking" were grown in the greenhouses from seed in polyethylene pots 25 cm in diameter and 23 cm deep with drainage holes in the bottom. Soil pH of the sterilized soil mixture was adjusted with limestone to 6.8. The plants were watered and fertilized as needed for optimum growth.

Artificial light from cool white fluorescent tubes insured a minimum of 3230 lux at a level of 1.1 m above pot height with a 16 hours photoperiod. Temperature averaged 23°C during the day and 16°C at night.

Four replications were used, each with six randomized treatments composed of 11 pots. Harvest was on the whole plant at appropriate maturity stages: early bud, 10% bloom and full bloom. The plants were cut about 1.5 cm from the crown. All the samples were frozen on dry ice and stored in a freezer at -20°C until used. The number of nodes on each plant was counted, starting from the node of the first fully expanded leaf near the shoot apex. In experiment I, plants were classified into ten groups of morphological development: 10, 12, 14, 16, 18, 20, 22, 24, 26 and 28 nodes.

Leaves and stems were separated on a piece of dry ice. The stem fraction included petioles and inflorescences wherever appropriate. The plant parts of the whole plant were placed separately on aluminum dishes and transfered into a cooler filled with dry ice. The

frozen samples were lyophilized to remove the moisture inside the plant material, dried and placed in a dessicator. The dried sample of leaf and of stem were weighed. The ratio of leaf to stem of the whole plant was then determined on a dry weight basis.

In experiment II, plants with 18 nodes were selected to evaluate the rate of changes of the leaf-stem ratio according to position on the shoot and maturity stages within and between both legumes. In addition, this selected sampling was used to establish the distribution of leaf and stem according to vertical layers and the repartition of different leaf types as well as stem types and inflorescence along the shoot layers and inside of each layer. The selected plants represented the median groups of plant development that had a high frequency in a plant population distribution at every maturity stage for both species.

These plants were sectioned into three segments of six nodes each: top, middle and bottom. The vertical layer sampling procedure minimized variations due to uncontrollable experimental factors and insured the same relative maturity of each plant layer. These three layers were obtained by dividing the total nodes of the whole plant by three and starting to count from node of the first fully expanded leaf down the plant. The top, the middle and the bottom were, respectively, the first to sixth node, the seventh to twelfth node, and the thirteenth to eighteenth node.

Branches made up of secondary-tertiary leaves and stems were cut, individually wrapped from the main shoot which represented the primary structure and placed in a freezer at -20^{o}C. The various plant fractions (primary leaves, secondary-tertiary leaves, primary stems plus petioles, secondary-tertiary stems plus petioles, inflorescences) were separated on

a piece of dry ice and placed separately into aluminum dishes that were transfered into a cooler of dry ice. The frozen samples were then lyophilized to remove the moisture inside of plant material.

The dried material was removed from the lyophilizer, transfered into a dessicator and the dry weight was taken for each of the samples. Data were first reported as the leaf-stem ratio for each vertical layer. The leaf fraction was composed of primary, secondary-tertiary stem and inflorescences when appropriate. The petioles from the primary leaves and from the secondary-tertiary leaves were included in their corresponding stem fraction.

Finally, the contribution of leaves and stems to the total plant leaves and stems was measured for each studied vertical layer. All reported data are on a dry matter basis.

The analysis of variance of all data in experiment I followed a 2 x 10 factorial arrangement with two replications. In experiment II and III, a 2 x 2 x 3 factorial with four replications was used on each plant parts (leaf, stem) for the analysis of variance. A Duncan's Multiple Range test was used on data of both experiments in order to determine the difference between the treatments. In addition, a t-test was used to determine the difference among the various treatments between alfalfa and birdsfoot trefoil.

RESULTS AND DISCUSSION

EXPERIMENT I

Leaf-stem ratio vs studied variables

The partition of the morphological components was affected differently by the level of plant development of each species (Table 1). The leaf-stem ratio increased significantly for alfalfa but not for trefoil as the plant developed (Table 2). Under the experimental conditions in the greenhouses, the faster growth of alfalfa led to a highly developed alfalfa aerial portion. Consequently, a greater variation in the partition of the leaf and stem occurred on alfalfa than on trefoil.

Stages of maturity did not affect the leaf-stem ratio of alfalfa but had a significant effect on trefoil (Table 1).

Leaf-stem vs maturity stages

The leaf-stem ratio of trefoil declined significantly from early bud (0.68) to full bloom (0.47) (Table 3). The leaf-stem ratio decreased with age in plants with 18, 20, 22 nodes (Table 2). Younger plants with 10, 12, 14, 16 nodes had a higher ratio at the early bud at the two blooming stages.

Leaf-stem ratio vs plant development

The mean leaf-stem ratio of both legumes at the two lowest groups of plant development(10 and 12 nodes) was lower than those of plants with the highest development (24, 26 and 28 nodes) (Table 2).

Within the early bud stage, alfalfa with 26 nodes showed a leaf-stem ratio of 0.77, higher than those reported on plants with 12 to 16 nodes (Table 2). At 10% bloom alfalfa with 24 nodes had the higest leaf-stem ratio (0.88). Alfalfa in full flower had a higher leaf-stem ratio on plants

Table 1. Analysis of variance of the factors; maturity stages and plant development of both alfalfa and birdsfoot trefoil and the combined factor interactions on the leaf-stem ratio (Exp. I).

Sources	Leaf-stem ratio	
	Alfalfa	Trefoil
Maturity stages	N.S.	**
Plant development	*	N.S.
Maturity stages x plant development	N.S.	N.S.

The significance of factors was evaluated at 1 percent level and followed by ** and by * at 5 percent level and N.S. followed those which are not significant.

Table 2. Variation in the partition of leaf to stem among the various morphological groups of two legumes at three different maturity stages (Exp. I).

Species	Development sta. / Maturity stages	10	12	14	16	18	20	22	24	26	28
Alfalfa	Early bud	0.69 ab	0.65 b	0.64 b	0.64 b	0.71 ab	0.74 ab	0.74 ab	0.75 ab	0.77 a	0.74 ab
	10% bloom	0.60 b	0.61 b	0.62 b	0.67 b	0.64 b	0.68 b	0.64 b	0.88 a	0.64 b	0.73 b
	Full bloom	0.55 b	0.59 b	0.61 ab	0.61 ab	0.63 ab	0.66 ab	0.64 ab	0.72 a	0.61 ab	0.69 ab
	Average	0.61 b	0.62 b	0.63 b	0.64 b	0.66 ab	0.69 ab	0.67 ab	0.78 a	0.67 ab	0.69 ab
Trefoil	Early bud	0.65 ab	0.61 b	0.64 ab	0.70 ab	0.67 ab	0.72 ab	0.66 ab	0.76 ab	0.78 a	0.62 b
	10% bloom	0.46 b	0.53 ab	0.50 ab	0.54 ab	0.56 a	0.57 a	0.56 a	0.52 ab	0.60 a	0.56 a
	Full bloom	0.42 b	0.40 b	0.47 ab	0.48 ab	0.43 ab	0.49 ab	0.46 ab	0.53 a	0.54 a	0.52 a
	Average	0.51 a	0.51 a	0.54 a	0.57 a	0.56 a	0.59 a	0.56 a	0.60 a	0.64 a	0.60 a
Average		0.56 D	0.56 D	0.58 CD	0.61 BCD	0.61 BCD	0.64 ABC	0.62 BCD	0.69 A	0.65 ABC	0.66 AB

Any two morphological groups within the same maturity stage and the average with the same letter are not significantly different at the 5 percent level by the Duncan's Multiple Range test.

Table 3 The mean ratio leaf to stem of legume species at each maturity stage (Exp.I).

Species	Early Bud	10% Bloom	Full Bloom	Average
Alfalfa	0.71 a	** 0.69 a	** 0.63 a	** 0.68
Trefoil	0.68 a	** 0.54 b	** 0.47 c	** 0.56
Average	0.69 A	0.61 AB	0.55 B	----

- Comparisons between maturity stages for the same species are shown by small letters and the means with the same letter are not significantly different at the 5 percent level by the Duncan's Multiple Range test.
- Comparisons between maturity stages for the bulk data of both species are shown by capital letters and the means with the same letter are not significantly different at the 5 percent level by the Duncan's test.
- Comparisons between both species are done by the t-test and the means that showed difference at the 1 percent level are followed by ** .

ratio (0.88). Alfalfa in full flower had a higher leaf-stem ratio on plants with 24 nodes than those with 10 and 12 nodes. Two hypothesis could be suggested to explain these results. Firstly, at the same maturity stage, as alfalfa increased its physiological age its leafiness enhanced following the development of numerous axillary buds. Secondly, the increasing leafiness of alfalfa with its advancing physiological age might be caused by the expansion of the existing secondary-tertiary leaves.

EXPERIMENT II

Leaf-stem ratio of plants with 18 nodes

The leaf-stem ratio of plants with 18 nodes was affected by all factors studied (Table 4) and by the species x maturity stages interaction.

Species

The leaf-stem ratio was greater on alfalfa (0.66) than on trefoil (0.56) (Table 5), possibly reflecting the greater vigor of alfalfa under the experimental conditions. This statement was supported by the studies of Bjorkman and Holmgren (1963) and Rhykerd et al (1959). Bjorkman and Holmgren found that plants in a greenhouse showed particularities in their physiological as well as morphological responses to the low light intensity. Rhykerd et al (1959) found that alfalfa and trefoil responded differently to given light intensities. Conversely, alfalfa showed a high leaf-stem ratio at low intensity but a lower ratio at high light intensities. They indicated that the lack of competiveness of trefoil could be associated with its low proportion of leaves under low light intensities.

Table 4. Analysis of variance of factors: species, maturity stages and plant layers and their interactions on the ratio leaf to stem, on the distribution of leaves and stems along the shoot of plant with intermediate number of node (18) (Exp. II and III).

Sources	Variables		
	Leaf-stem ratio	Distribution of leaves	Distribution of stems
Species	**	N.S.	N.S.
Maturity stages	**	N.S.	N.S.
Plant layers	**	**	**
Species x maturity stages	N.S.	N.S.	N.S.
Species x plant layers	N.S.	**	**
Maturity stages x plant layers	**	**	**
Species x maturity stages x plant layers	N.S.	**	**

The significance of factors was evaluated at 1 percent level and followed by ** and by * at 5 percent level and N.S. followed those which are not significant.

Table 5. The mean leaf-stem ratio of each legume species with an intermediate number of nodes at each studied maturity stages (Exp. II).

Species	Early Bud	10% Bloom	Full Bloom	Average
Alfalfa	0.71 a	** 0.64 b	** 0.63 b	* 0.66
Trefoil	0.67 a	** 0.56 b	** 0.43 c	* 0.56
Average	0.69 A	0.60 B	0.53 B	----

- Comparisons between maturity stages for the same species are shown by small letters and the means with the same letter are not significantly different at the 5 percent level by the Duncan's Multiple Range test.
- Comparisons between maturity stages for the bulk data of both species are shown by capital letters and the means with the same letter are not significantly different at the 5 percent level by the Duncan's test.
- Comparisons between both species are done by the t-test and the means that showed difference at the 5 percent level are followed by * and by ** at the 1 percent level.

Maturity stages

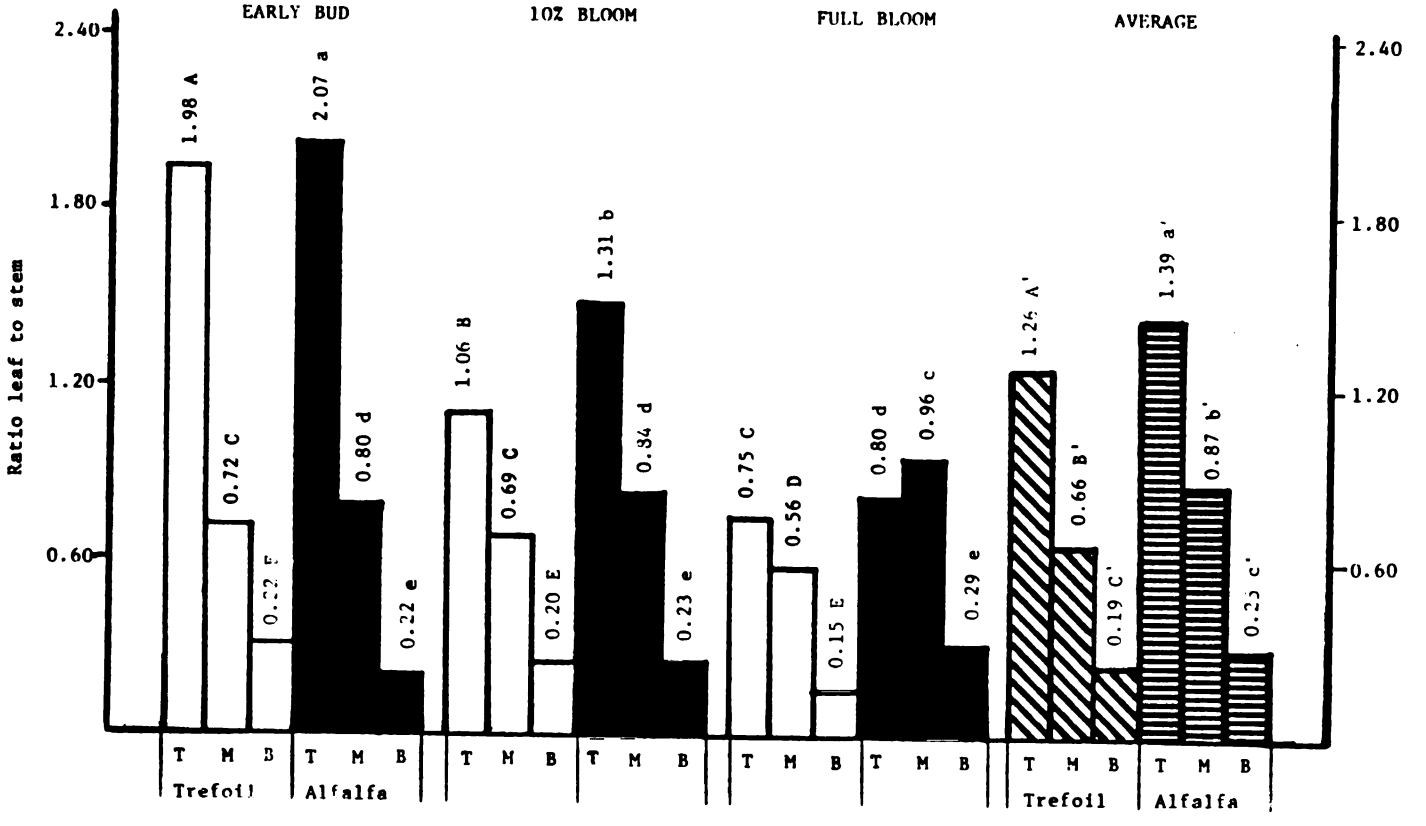
The leaf-stem ratio of alfalfa and trefoil decreased between early bud to 10% bloom (Table 4) but only trefoil showed a decline from 10% bloom (0.56) to full bloom (0.43). Most authors report a decline in the leaf-stem ratio of alfalfa with age due to the increase of steminess in the whole plant (Kiesselbach and Anderson 1926; Mac Donald 1946; Terry and Tilley 1964; Mowat et al 1965). No information is available on the decrease of leaf-stem ratio of trefoil with increasing maturity but a similar explanation as for alfalfa is suggested.

Vertical layers

The leaf-stem ratio decreased from the top (1.39) to the bottom (0.25) on alfalfa and from 1.26 to 0.19 on trefoil (Fig. 1). The leaf-stem ratio declined for each species and for each maturity stage (Fig. 1). The increase in stem weight down the shoot was a major factor in the decline of the ratio along the shoot in earlier studies (Terry and Tilley 1964; Fuess and Tesar 1968; Smith 1970; Thom 1978). Falling leaves (Rhykerd et al 1959; Verhagen et al 1963; Taylor and Templeton 1966; Fuess and Tesar 1968; Carter and Scheaffer 1983) and small leaves in the shadow of the canopy (Cooper and Qualls 1967) reduce the leaf fraction down the stem.

Comparison of the leaf-stem ratio between species

Full bloom alfalfa was 50% leafier with a leaf-stem ratio of 0.29 than trefoil (0.15) at the basal segment. In the upper layer, the leaf-stem ratio declined with maturity from 2.07 to 0.80 on alfalfa and 1.98 to 0.75 on birdsfoot trefoil (Fig. 1). Both legumes had, however, a similar ratio at the top shoot at every maturity stage. The results suggest that both



Vertical layers T: Top (1-6 nodes) M: Middle (7-12 nodes) B: Bottom (13-18 nodes)

Figure 1. Vertical and horizontal distribution of the leaf-stem ratio within both alfalfa and birdsfoot trefoil (Exp. II).

Comparisons between layers and maturity stages are shown by capital letter within trefoil and by small letter within alfalfa and the means with the same letter are not different at the 5 percent level by the Duncan's Multiple Range test.

Comparisons between layers of the bulk data within each species are shown by capital letter with ' for trefoil and by small letters with ' for alfalfa.

species have a comparable sink strenght for developing new tissues. As the plant matures, the contribution of blooms increases into the stem fraction (Table 6) leading to a decline of the upper leaf-stem ratio.

In the middle layer, the leaf-stem ratio of trefoil decreased from 0.72 at early bud and 0.69 at 10% bloom to 0.56 at full bloom (Fig. 1). Conversely, the alfalfa leaf-stem ratio increased from 0.80 at early bud to 0.84 at 10% bloom and 0.96 at full bloom. The above results were likely related to the amount of second and third order structures that occurred in that layer on both legumes at full flower. The median portion of alfalfa had four times more secondary-tertiary leaves and two times more secondary-tertiary stems than on median portion of trefoil (Table 7 and 8). In addition, with increasing maturity, the contribution of the second and third order stems to the whole plant stem remained unchanged at the control portion of both legumes (Table 7).

The above finding tends to support two explanations. Firstly, the increase of the leaf-stem ratio of alfalfa with increasing maturity probably came from the full expansion of the secondary-tertiary leaves as the plant reached full bloom stage giving a proportion of leaves that was very close to the proportion of the stem at full bloom. Secondly, the increasing weight of the trefoil stem fraction could be attributed to the increase of inflorescences as well as to the strength of the trefoil stem to accumulate nutrients as the plant aged. As a result, the increasing stem weight over came the contribution of secondary-tertiary leaves to the total amount of leaves and may explain the decreasing leaf-stem ratio with age.

Table 6. Vertical changes in the percentage contribution of flowers to the total plant stems fraction in both alfalfa and birdsfoot trefoil at three maturity stages (Exp. II).

Species	Layers-Nodes	Maturity Stages			
		Early bud	10% Bloom	Full Bloom	Average
Alfalfa	Top (1-6)	0 d	5 b	12 a	6 A
	Middle (7-12)	0 d	0 d	2 c	1 B
	Bottom (13-18)	0 d	0 d	1 d	0 B
	Average	0 B	2 B	5 A	2
Trefoil	Top (1-6)	0 d	11 b	13 a	8 A
	Middle (7-12)	0 d	0 d	3 c	1 B
	Bottom (13-18)	0 d	0 d	0 d	0 B
	Average	0 B	4 A	5 A	3
Average		0 B	3 A	5 A	-

Comparisons between layers and maturity stages within each species are shown by small letters and the means with the same letter are not different at the 5 percent level by the Duncan's Multiple Range test.

Comparisons between maturity stages and between layers within each species are shown by capital letter and the means with the same letter are not different at the 5 percent level by the Duncan's test.

Table 7. Vertical changes in the percentage contribution of secondary-tertiary leaves to the total plant leaves of both alfalfa and birdsfoot trefoil at three maturity stages (Exp. II).

Species	Layers-Nodes	Maturity Stages			
		Early Bud	10% Bloom	Full Bloom	Average
Alfalfa	Top (1-6)	7 c	5 cd	2 d	5 B
	Middle (7-12)	24 b	25 ab	28 a	25 A
	Bottom (13-18)	2 d	3 d	6 cd	3 B
	Average	13 A	11 A	12 A	12 **
Trefoil	Top (1-6)	1 c	1 c	1 c	1 B
	Middle (7-12)	6 ab	5 b	7 a	6 A
	Bottom (13-18)	2 c	1 c	2 c	1 B
	Average	3 A	2 A	3 A	3 **
Average		8 A	6 A	7 A	-

Comparisons between layers and maturity stages within each species are shown by small letters and the means with the same letter are not different at the 5 percent level by the Duncan's Multiple Range test.

Comparisons between maturity stages and between layers within each species are shown by capital letter and the means with the same letter are not different at the 5 percent level by the Duncan's test.

Comparisons between both species (average) are done by the t-test and the means that shown difference at the 1 percent level are followed by ** .

Table 8. Vertical changes in the percentage contribution of secondary-tertiary stems to the total plant stems in both alfalfa and birdsfoot trefoil at three maturity stages (Exp. II).

Species	Layers-Nodes	Maturity Stages			
		Early Bud	10% Bloom	Full Bloom	Average
Alfalfa	Top (1-6)	1 bc	1 bc	0 c	1 B
	Middle (7-12)	6 a	6 a	7 a	6 A
	Bottom (13-18)	1 bc	1 bc	2 b	1 B
	Average	3 A	2 A	3 A	3
Trefoil	Top (1-6)	0 c	0 c	0 c	0 C
	Middle (7-12)	4 a	2 b	3 ab	3 A
	Bottom (13-18)	3 ab	1 c	1 c	2 B
	Average	2 A	1 A	1 A	2
Average		3 A	1 A	2 A	-

Comparisons between layers and maturity stages within each species are shown by small letters and the means with the same letter are not different at the 5 percent level by the Duncan's Multiple Range test.

Comparisons between maturity stages and between layers within each species are shown by capital letter and the means with the same letter are not different at the 5 percent level by the Duncan's test.

EXPERIMENT III

Distribution of leaves on selected plants with 18 nodes

Within each species, the distribution of the whole plant leaf weight was greatest in the middle layer following by the top and bottom layer (Fig. 2). The weight of leaf at the middle stem portion was 55% of the total on alfalfa and 45% on trefoil. This finding was in agreement with those of Smith (1970) and Bula (1972) who both found that most alfalfa leaves were near the middle of the plant. The above result could be linked to some morphological characteristics such as leaf shape (the ratio of length to breadth became greater for leaves at the central layer) and leaf development (leaves from development of axillary buds).

Leaves from the upper shoot segment represented 42% of trefoil and 30% of the alfalfa leaves (Fig. 2). The low contribution of basal to total leaves (12% of trefoil and 15% of alfalfa) was mainly due to dropping of the lower leaves as shown on alfalfa by Fuess and Tesar (1968).

Comparison of leaf distribution between both species

The percentage of leaves between both legumes showed significant differences in the vertical layers as well as in the maturity stages studied (Fig. 2). At every maturity stage, trefoil was more leafy than alfalfa in the top layer. For instance, at full bloom, the proportion of upper leaves of trefoil was almost twice that of alfalfa. Trefoil was more uniform than alfalfa in its leaf distribution between the two upper shoot segments with increasing maturity (Fig. 2).

The upper-central leaves on alfalfa declined from 89 to 81% of the total while on trefoil the leaves remained constant near 88%.

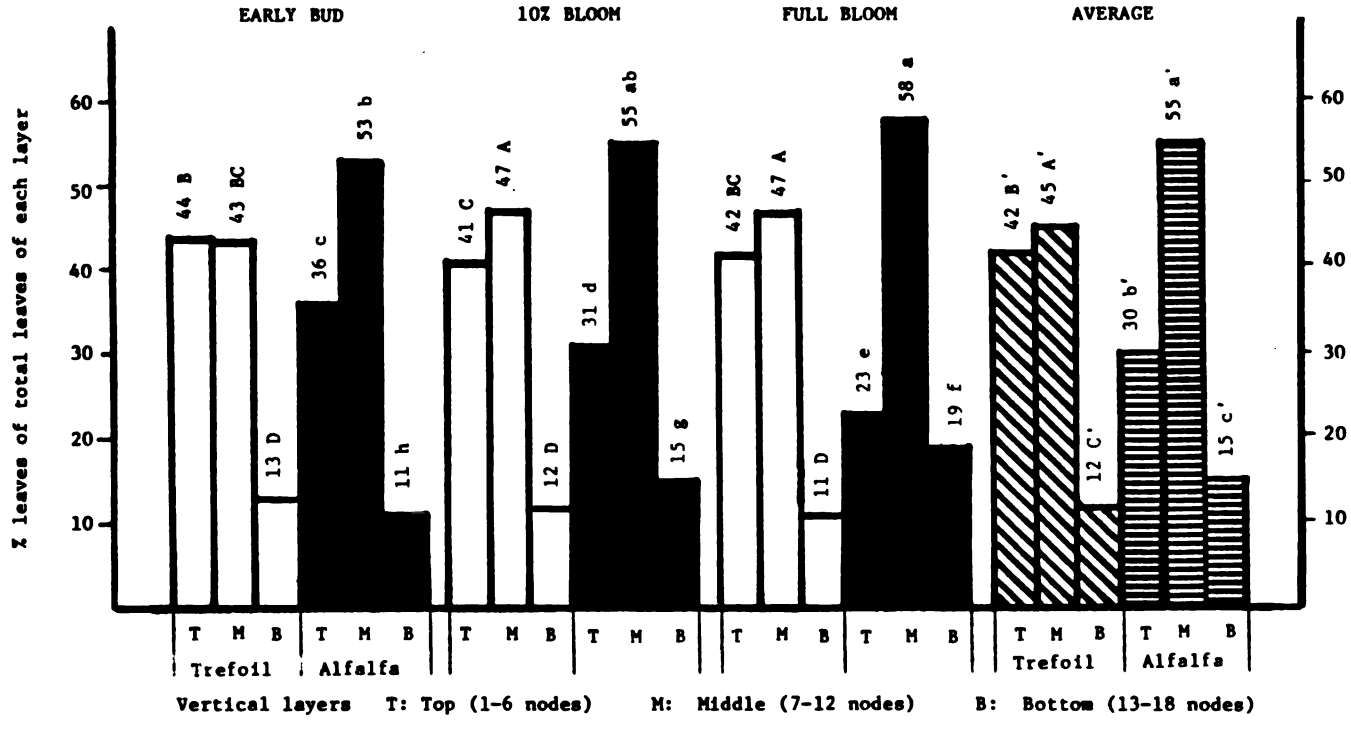


Figure 2. Vertical changes in the contribution of leaves to the whole plant leaves in alfalfa and birdsfoot trefoil at various maturity stages (Exp. III).

Comparisons between layers and maturity stages are shown by capital letter within trefoil and by small letter within alfalfa and the means with the same letter are not different at the 5 percent level by the Duncan's Multiple Range test.

Comparisons between layers of the bulk data within each species are shown by capital letter with ' for trefoil and by small letters with ' for alfalfa.

The decline in alfalfa leaves was mainly due to the decrease from 36 to 23% in upper leaves as it matured (Fig. 2). Physiological events such as competition for water by the lower leaves or remobilization of nutrients toward the floral organs could occur as alfalfa increased in maturity and could explain the decline.

The occurrence of few branches at the basal portion of alfalfa contributed to the increase of leaves from 11 to 19% of the total as the plant advanced in age (Fig. 2).

Stem distribution

Neither species nor stages of maturity influenced stem distribution. Most combined factor interactions, except species x maturity stages, significantly affected the distribution of stem along the shoot.

Vertical layers

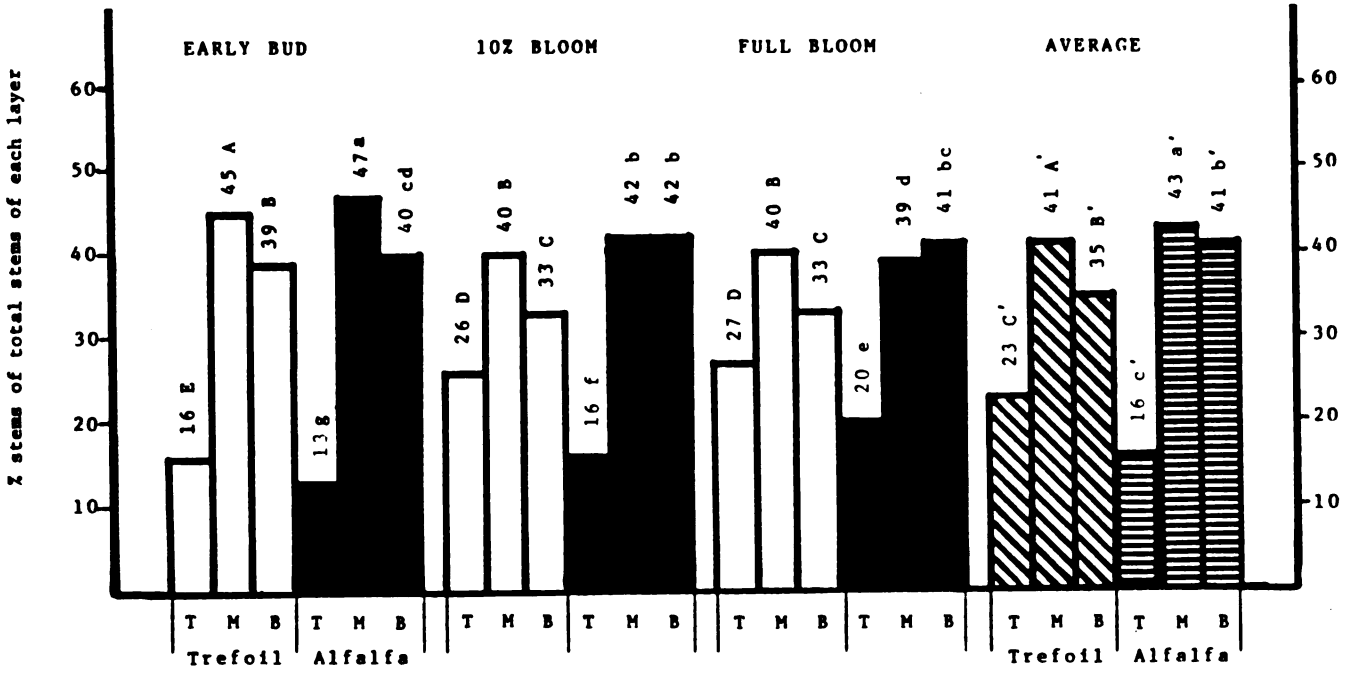
Both legumes showed a similar pattern of stem distribution, decreasing in order from middle, bottom to the top layer (Table 9). The largest percentage of stem, as for the leaves, of the total stem was at the central portion of both alfalfa (43%) and trefoil (41%) (Fig. 3). The presence of a vascularization high in conducting water tissues (Chapter II) and a high light intensity would favor the concentration of new tisuue at the median shoot portion.

Although the degree of vascularization of the basal stem was similar at the median stem (Chapter II), little light reached the bottom layer of the canopy resulting in little development of axillary buds. The percentage of basal stem of the plant stem was greater on alfalfa (41%) than on trefoil (35%) (Fig. 3).

Table 9. Mean contribution of leaves (%) and of stem (%) of each plant layer to the whole plant leaf and stem of the bulk data of both leguminous species (Exp. III).

Plant parts	Top (1-6 nodes)	Middle (7-12 nodes)	Bottom (13-18 nodes)
Leaves	36 b	50 a	14 c
Stems	20 c	42 a	38 b

Comparisons between layers on the bulk data for each plant part are shown by small letters and the means with the same letter are not significantly different at the 5 percent level by the Duncan's Multiple Range test.



Vertical layers T: top (1-6 nodes) M: middle (7-12 nodes) B: Bottom (13-18 nodes)

Figure 3. Vertical changes in the contribution of stems to the whole plant stems in alfalfa and birdsfoot trefoil at various maturity stages (Exp. III).

Comparisons between layers and maturity stages are shown by capital letter within trefoil and by small letter within alfalfa and the means with the same letter are not different at the 5 percent level by the Duncan's Multiple Range test.

Comparisons between layers of the bulk data within each species are shown by capital letter with ' for trefoil and by small letters with ' for alfalfa.

Compared to trefoil, alfalfa showed a more uniform distribution of stem between the middle and the bottom layers, possibly explaining the greater rigidity of the alfalfa shoot compared to trefoil which lodges more easily than alfalfa. Trefoil was more stemmy than alfalfa at the top segment (Fig. 3) coinciding with the greater leafiness of trefoil over alfalfa in the upper layer.

Stem partition vs species, maturity stages and plant layers

As plants matured, important changes in the proportion of stem occurred at various layers. The upper stem portion of both legumes increased from early bud (13%) to full bloom (20%) (Fig. 3). The contribution of blooms became a major constituent of the stem fraction as the plant blossomed. From early bud to 10% bloom, the stem portion of birdsfoot trefoil increased more than on alfalfa, probably because trefoil has a long and thick peduncle supporting the inflorescence. Moreover, the decline of the stem proportion at the median portion between early bud to blooming stages (Fig. 3) of both species could be an indication of a narrowing shoot, particularly on alfalfa where the median stem diameter declined from early bud (0.156 cm) to full bloom (0.120 cm) (Table 10). The decline of the central stem fraction of trefoil as it matured was not associated with a smaller stem diameter as on alfalfa but possibly as a remobilization of nutrients to the floral organs.

At the basal layer, plants at 10% bloom were more stemmy than on those at early bud. At full bloom, the contribution of stem to the total stem was intermediate between the two first studied growth stages.

Table 10. Vertical changes of the stem diameter of both alfalfa and birdsfoot trefoil at three maturity stages.

Species	Layers	Maturity Stages			
		Early Bud	10% Bloom	Full Bloom	Average
		(cm)	(cm)	(cm)	(cm)
Alfalfa	Top (4th node)	0.126 bc	0.108 cd	0.099 d	0.111 B
	Middle (9th node)	0.156 a	0.147 ab	0.120 cd	0.141 A
	Bottom (15th node)	0.153 ab	0.155 a	0.130 abc	0.146 A
	Average	0.145 A	0.137 A	0.117 B	0.133 **
Trefoil	Top (4th node)	0.089 c	0.090 c	0.082 c	0.098 B
	Middle (9th node)	0.116 ab	0.111 b	0.118 ab	0.115 A
	Bottom (15th node)	0.135 ab	0.120 ab	0.131 ab	0.127 A
	Average	0.113 A	0.107 A	0.111 A	0.113 **
Average		0.129 A	0.122 A	0.114 A	-----

Comparisons between layers and maturity stages within each species are shown by small letters and the means with the same letter are not different at the 5 percent level by the Duncan's Multiple Range test.

Comparisons between maturity stages and between layers within each species are shown by capital letter and the means with the same letter are not different at the 5 percent level by the Duncan's test.

Comparisons between both species (average) are done by the t-test and the means that shown difference at the 1 percent level are followed by ** .

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CHAPTER II

HISTOLOGICAL STUDY
OF
ALFALFA AND BIRDSFOOT TREFOIL

ABSTRACT

Alfalfa (*Medicago sativa L.*) and birdsfoot trefoil (*Lotus corniculatus L.*) grown in the greenhouse in a soil mixture corrected for pH and fertility were sampled at an 18 node stage when they reached early bud, 10% bloom and full bloom. A central leaflet and the internode stem taken from the fourth, ninth and fifteenth node were used to determine the anatomical components within and between species and to evaluate changes along the shoot.

Leaf anatomy was greatly affected by the species. The percentage area of vascular bundles, sclerenchyma, xylem and phloem was greater in the leaves of alfalfa than in trefoil. Mesophyll cells area was greater in trefoil than in alfalfa. The epidermis and collenchyma were similar in both legumes. Stage of maturity did not have any influence on the leaf anatomy of each species.

Leaf position on the stem did not influence the percentage area of most anatomical components. Epidermis increased and mesophyll cells decreased from plant top to bottom. Percentage area of epidermis and leaf thickness were negatively correlated. Leaf thickness and the percentage of mesophyll cells area were positively correlated.

The area of most leaf anatomical components and leaf *in vitro* dry matter digestibility (IVDMD) of both legumes was not correlated. The amounts of epidermis, sclerenchyma and mesophyll cells were significantly correlated with the relative feeding value (RFV). The area of epidermis and the leaf RFV were positively correlated on alflafa ($r = +0.62$) and trefoil ($r = +0.61$). The area of mesophyll cells was negatively correlated

with the leaf RFV of both alfalfa (r= - 0.62) and trefoil (r= - 0.50). Trefoil leaf RFV and the area of sclerenchyma were negatively correlated (r= - 0.42). Trefoil had a greater percentage area of epidermis, chlorenchyma, sclerenchyma and interfascicular xylem than alfalfa.

The xylem:phloem ratio was higher for alfalfa (4:1) than trefoil (3:1). Trefoil had more sclerenchyma area in its stem than did alfalfa. The ratio of the area of sclerenchyma to collenchyma was higher on trefoil (12:1) than on alfalfa (3:1).

The epidermis, bundle sheath, interfascicular xylem, vascular bundle and pith parenchyma cells were statistically affected by the position on the stem. The area of epidermis decreased from 6.9 to 5.4% down the trefoil shoot. In alfalfa the area of epidermis, bundle sheath and pith parenchyma decreased from the top to the middle segment and then remained constant. The area of interfascicular xylem and of vascular bundles increased from the top to middle segments and then remained stable.

The pith parenchyma cells, bundle sheath and epidermis of alfalfa and trefoil and chlorenchyma, collenchyma and phloem for alfalfa were positively correlated with stem digestibility. The area of vascular bundles and xylem in both legumes and of sclerenchyma in trefoil, and of interfascicular xylem in alfalfa were negatively correlated to the IVDMD and to the RFV of the stem.

INTRODUCTION

Research has recently been conducted on the histology of leaf and stem of forage species to determine if they had some effects on digestibility and intake (Schank et al 1973, Akin and Burdick 1975 and Swakon and Moore 1980).

Swakon and Moore (1980) introduced a general pattern to describe leaf and stem anatomy of most forage species. The leaf consisted of: a epidermal layer covered with a cuticle; a high proportion of mesophyll cells (palisade and spongy cells); and a vascular system involving a large midrib bundle and smaller secondary veins and mechanical tissues with varying degree of lignification. The stem consisted of: epidermis covered with a cuticle; cortex either or both collenchyma and sclerenchyma; lignified vascular bundles surrounded by bundle sheath; and a central parenchyma cells (pith).

Some researchers have shown that species, maturity stage and position on the shoot could quantitatively affect the distribution of the above anatomical components of leaf and stem. Wilkins (1972), Schank et al (1973), Akin and Burdick (1975) and Cohen et al (1982) estimated the amount of various anatomical structures in plant parts. Area were measured by photomicrographs projected on graph paper (Wilkins 1972 and Akin and Burdick 1975) or on a screen (Hanna et al, 1973). Others traced an outline of the tissues from camera lucida (Schank et al 1970, Cohen et al 1982) or from a planimeter (Wilson and Davis 1976). They determined the tissue areas by counting the number of squares on the graph paper, by calculating the geometric figures of each tissue,

by weighing the paper, or by using a planimeter.

Using some of these methods, Akin and Burdick (1975) reported differences in the amount of some tissue types among grass species. For instance, the cross section of orchardgrass (*Dactylis glomerata L.*) leaf blade was 65 to 70 percent of living tissues such as mesophyll cells, phloem, parenchyma bundle sheath. The same tissue types in leaf blades of bermudagrass (*Cynodon dactylon L.*) represented only 30 percent of the cross sectional area.

Maturity stages affect the distribution of various tissue types among species, controversery exists over that statement. Wilkins (1972) found, that with maturity, there was variation in the amount of sclerenchyma and vascular bundles in both the leaf blades and leaf sheath of the studied grass species. Many authors, however, found that the percent of the total cross sectional area of most tissue types including vascular bundles did not show any significant change during maturation (Schank et al 1973, Hanna et al 1976).

Quantitative changes in the distribution of the different anatomical components of plant parts could also occur vertically within the plant. The only known study on that topic was by Akin et al (1977) on coastal bermudagrass. These authors found, from the top to the bottom of the plant, an increase in total vascular tissue and a decrease in mesophyll cell fraction; the amount of sclerenchyma in the leaf sheath declined.

The histological examination of grass aerial parts has always been the researcher's main concern. No extended study dealing with le-

gumes was found, explained, in part,by the high digestibility of legumes. A qualitative study, however, on the leaflet and stem anatomy of alfalfa was described many years ago by Winton (1914) as follows: the leaflet consists of: epidermis covered by a cuticle and by hairs; mesophyll cells with palisade cells distributed in two rows and spongy cells loosely arranged and air spaces; midvein forming a ridge on the abaxied surface ; bundle sheath surrounding the midvein, and mechanical tissues surrounding the midvein, and mechanical tissues surrounding the secondary veins. The stem consists of: epidermal cells longitudinally arranged in rows and covered with a cuticle; cortical cells frequently interrupted by masses of collenchyma; major vascular bundle located at the axis with the smallest one between axis; rows of parenchyma cells occurred between rows of xylem; and bundle sheath surrounding a zone of sclerenchyma.

The qualitative description of the leaflet and stem anatomy of birdsfoot trefoil generally followed the same description as mentioned above for alfalfa, with the particularity that the trefoil leaflet contained some vesicules of tannin in the mesophyll cells.

Differences in digestibility between the above two legumes has been reported by Horrocks and Washko (1968) and Allinson et al (1969). Both studies were, however, compared the digestibility of the whole plants.

The purposes of the present study were to: measure the amount of the various tissue types in the leaf and stem of alfalfa and birdsfoot trefoil; compare the distribution of anatomical components in both leaves and stems of both legumes according to maturity stages and vertical layers; and measure the degree of association of the various anatomical components in leaves and stems to the *in vitro* digestibility and relative feeding value of each plant part.

METHODOLOGY

Alfalfa and birdsfoot trefoil were grown in a greehouse as described in chapter I. Five plants of eighteen nodes each were randomly collected for each species at three maturity stages: early bud, 10% bloom and full bloom. Stages were chosen in order to determine changes in the anatomy of plant parts between an early growth and a matured stage. Ten percent bloom, which is a commonly recommended harvesting stage, was used to determine changes in the amount of tissues with maturity.

Stems with a specific number of nodes (18) were collected so as to limit variations between genotypes with varying morphological development. The stems were sectioned into three segments each with six nodes to determine changes in distribution of anatomical components with maturity and with position on the plant.

Samples of leaf and stem were taken from the fourth, the ninth and the fifteenth node, which represented the third internode of each segment. Penfound (1931) suggested a similar procedure because of the occurrence of the same type of variation in leaf structure from corresponding nodes. All of these morphological considerations were needed in order to get representative measurement due to no interaction of genotypes with age or with leaf or stem position as suggested by Jones (1979).

A 2 mm portion of the leaflet was cut at the middle point of the central leaflet and at the internode immediately above the selected node.

The sections were fixed in 5% glutaraldehyde in 0.1M cacodylate at pH 7.2 for several hours, dehydrated in 10, 20, 30, 40, 50, 70, 90 and 100 percent (3X) ethanol and then stored in a cool place (5^{o} C) in absolute

ethanol until used.

Specimens were gradually transfered from absolute ethanol into clearing agent 10, 20, 30, 40, 50, 70, 90 and 100 percent (3X) xylene. Subsequently, specimens were embedded in paraffin and kept in a cool place (5^{o} C) for further examination.

Transversal sections 10 and 15 μm thick were obtained with a rotary microtome on the leaflet and stem specimens, respectively. Sections were stained in two successive phases. They were placed in safranine for at least 12 hours, dipped in crystal violet for 30 seconds, rinsed with xylene, and permanently mounted on microscope slides. A total of 180 slides of leaflet and stem specimens was studied on a Zeiss photomicroscope III.

Five areas, representative of leaf structure, were sampled for each cross section of a leaflet: the midvein area, the two areas adjacent to the midvein, and the area between two major secondary vascular bundles which was approximately midway between the midvein and the leaf tip. A total of 450 cross sectional areas of leaflets was examined.

Four zones of the stem were sampled: two at the axial area and two at the interfascicular area. A total of 360 cross sectional areas of stems was examined.

Enlargement of 24 mm x 36 mm negatives was made on 134 mm x 202 mm photographic prints. The final magnification of the 810 cross sectional areas of leaflet and stem was 1434 X. Tissue types were identified, cut out with a pair of scissor and then measured with an electronic leaf area planimeter.

The amount of each tissue type was expressed as a percentage of the studied cross sectional leaf or stem area (total of 7110). The area of the mesophyll cell fraction of leaflet of alfalfa and trefoil included air space. The results were averaged and attributed to their respective treatments of species, maturity stages, and plant layers.

Leaf thickness and stem diameter were estimated by recording eight to 10 readings for the cross section of each leaf and stem. A total of 1810 measurements was made on a Wild microscope at a magnification of 60 X. The number of vascular bundles in the leaf and stem was counted on each of the 180 cross sections of alfalfa and trefoil.

In vitro dry matter digestibility (IVDMD) was determined according to a modified two stages technique of Tilley and Terry (1963) done by Marten and Barnes (1980) (Chapter IV). The feeding relative value (RFV) was determined as an estimate of its digestible dry matter intake (Chapter III) as proposed by Barnes et al (1977).

A factorial arrangement analysis was performed on all data. The Duncan Multiple Range test was used to differentiate treatments at $p < 0.05$. A t-test was used to determine if some significant difference occurred between alfalfa and birdsfoot trefoil.

RESULTS AND DISCUSSION

LEAVES

Effect of species, maturity and vertical layers on anatomical components

The percentage of the various leaf tissue types was highly affected by the species (Table 11). The finding is in agreement with those on grasses by Schank et al (1973) and Akin and Burdick (1975) who found that the species was important in affecting the percentage of the different tissues of the plant part.

The leaf of alfalfa had a significantly greater amount of vascular bundles than that of trefoil (Table 12). Consequently, the amount of sclerenchyma, xylem and phloem were greatly affected (Fig. 4, 5, 6). These results support those of Cooper (1966) who found that alfalfa had a greater leaf area than trefoil. It could then be assumed that a greater leaf area meant a larger number of vascular bundles (Table 13) and amount of mechanical tissues as well as a bigger area of vascular bundles (Fig. 4, 5, 7, 8).

The leaf cross section of trefoil had a greater percentage of mesophyll cells (69.9%) than the alfalfa leaf (64.5%) (Table 11). At every layer, more mesophyll cells were found in trefoil than in alfalfa (Fig. 9), in agreement with Howarth et al (1978) who found that the bloat-safe legume birdsfoot trefoil had a more mesophyll cells than bloat-causing alfalfa.

The amount of epidermis in the leaf area was comparable for both legumes (Table 11) but epidermal cells were larger in trefoil than in alfalfa leaves. The ratio of leaf length to leaf width in alfalfa (30.8) was almost twice as high as that of trefoil (16.2) (Table 13).

Table 11. Mean area of anatomical components in leaves of alfalfa and birdsfoot trefoil and analysis of variance of the main factors: species, maturity stages and plant layers on the percentage area of anatomical components (in plant with 18 nodes development).

Tissue Types	Species		Factors		
	Alfalfa	Trefoil	S	M	P
	(%)	(%)			
Epidermis	22.4	23.2	N.S.	N.S.	**
Mesophyll cells	64.5 **	69.9 **	**	N.S.	*
Collenchyma	2.8 **	1.8 **	N.S.	N.S.	N.S.
Vascular bundle	10.6 **	5.2 **	**	N.S.	N.S.
Sclerenchyma	3.2 **	0.8 **	**	N.S.	N.S.
Phloem	2.4 **	1.4 **	*	N.S.	N.S.
Xylem	5.2 **	3.1 **	**	N.S.	N.S.

The significance of factors was evaluated at 1 percent level and followed by ** and by * at 5 percent level and N.S. followed those which are not significant.

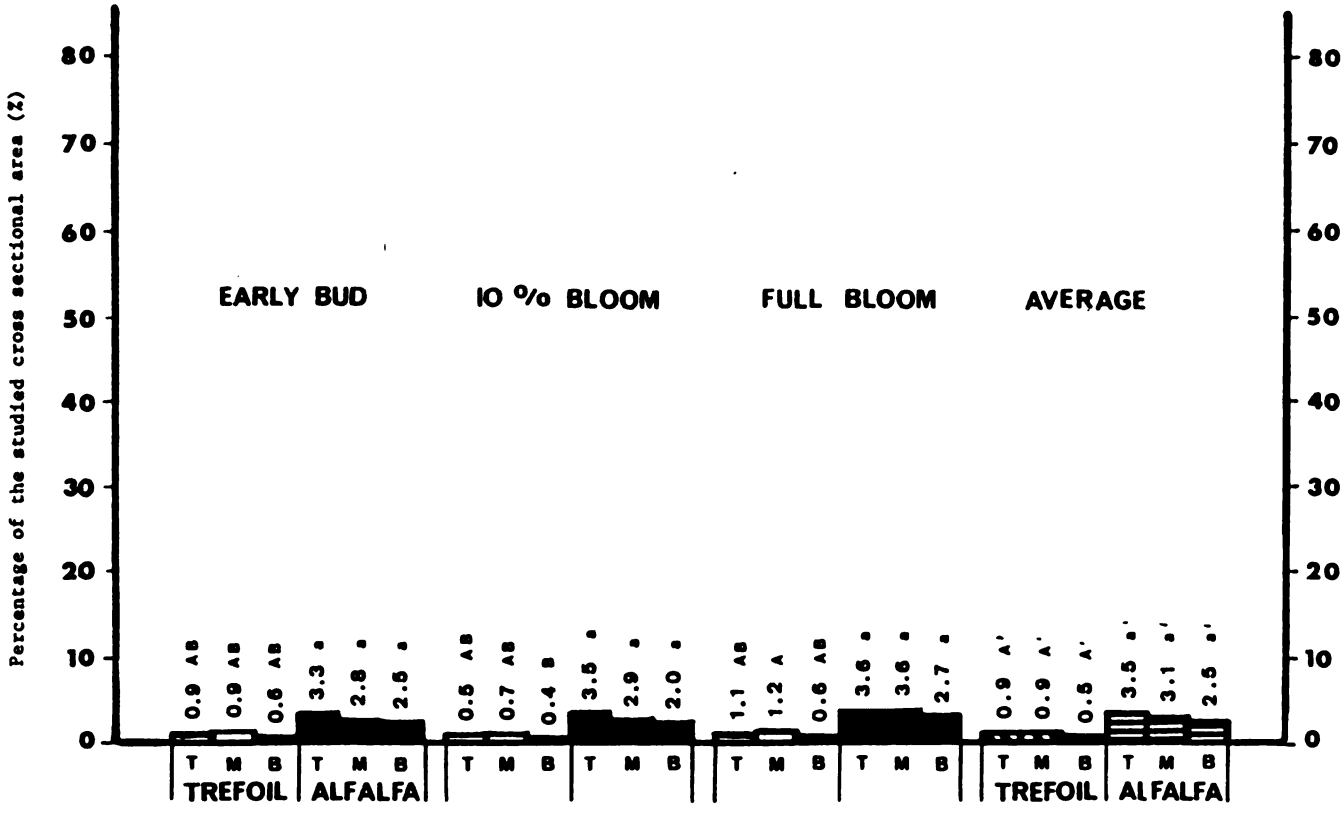
Table 12. Vertical distribution of the number of vascular bundles in leaf cross section of alfalfa and birdsfoot trefoil at three maturity stages.

Species	Layers	Maturity stages			
		Early bud	10% Bloom	Full bloom	Average
Alfalfa	Top (4th node)	21 ab	17 b	17 b	18 B
	Middle (9th node)	24 ab	25 a	21 ab	23 A
	Bottom (15th node)	22 ab	22 ab	23 ab	22 A
	Average	22 A	21 A	20 A	21 **
Trefoil	Top (4th node)	16 a	14 a	12 a	14 A
	Middle (9th node)	12 a	13 a	14 a	13 A
	Bottom (15th node)	9 a	10 a	14 a	11 A
	Average	12 A	12 A	13 A	13 **
Average		17 A	16 A	16 A	—

Comparisons between layers and maturity stages within each species are shown by small letters and the means with the same letter are not different at the 5 percent level by the Duncan's Multiple Range test.

Comparisons between maturity stages and between layers within each species are shown by capital letter and the means with the same letter are not different at the 5 percent level by the Duncan's test.

Comparisons between both species (average) are done by the t-test and the means that shown difference at the 1 percent level are followed by ** .

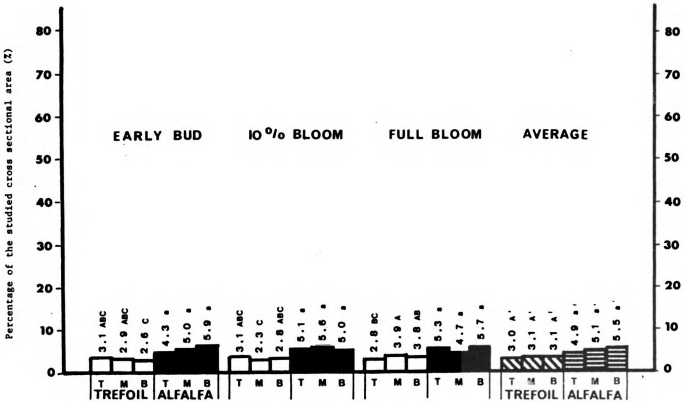


Vertical layers T: top (4^{th} node) M: middle (9^{th} node) B: bottom (15^{th} node)

Figure 4. A vertical distribution of sclerenchyma in leaves of alfalfa and birdsfoot trefoil at various maturity stages.

Comparisons between layers and maturity stages are shown by capital letter within trefoil and by small letter within alfalfa and the means with the same letter are not different at the 5 percent level by the Duncan's Multiple Range test.

Comparisons between layers of the bulk data within each species are shown by capital letter with ' for trefoil and by small letters with ' for alfalfa.

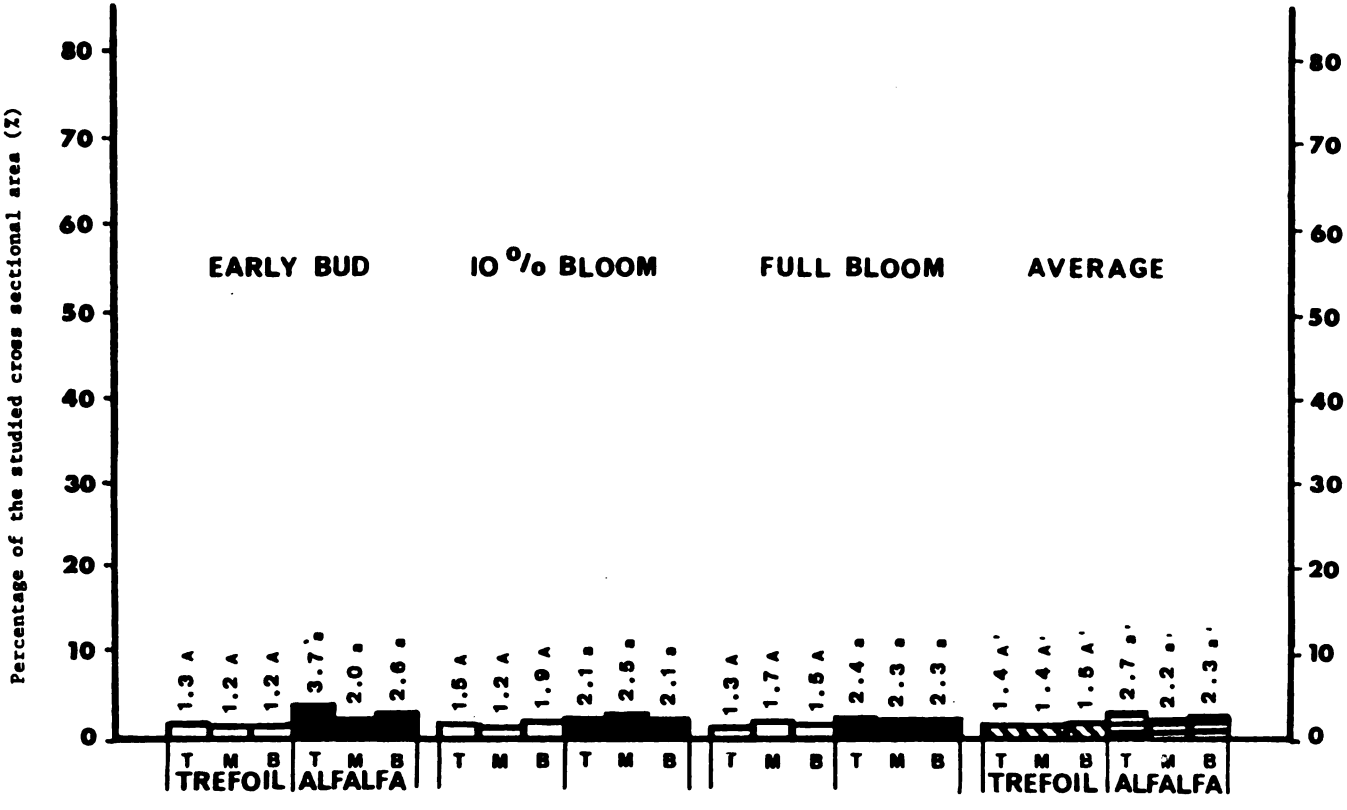


Vertical layers T: top (4th node) M: middle (9th node) B: bottom (15th node)

Figure 5. A vertical distribution of xylem in leaves of alfalfa and birdsfoot trefoil at various maturity stages.

Comparisons between layers and maturity stages are shown by capital letter within trefoil and by small letter within alfalfa and the means with the same letter are not different at the 5 percent level by the Duncan's Multiple Range test.

Comparisons between layers of the bulk data within each species are shown by capital letter with ' for trefoil and by small letters with ' for alfalfa.



Vertical layers T: top (4th node) M: middle (9th node) B: bottom (15th node)

Figure 6. A vertical distribution of phloem in leaves of alfalfa and birdsfoot trefoil at various maturity stages.

Comparisons between layers and maturity stages are shown by capital letter within trefoil and by small letter within alfalfa and the means with the same letter are not different at the 5 percent level by the Duncan's Multiple Range test.

Comparisons between layers of the bulk data within each species are shown by capital letter with ' for trefoil and by small letters with ' for alfalfa.

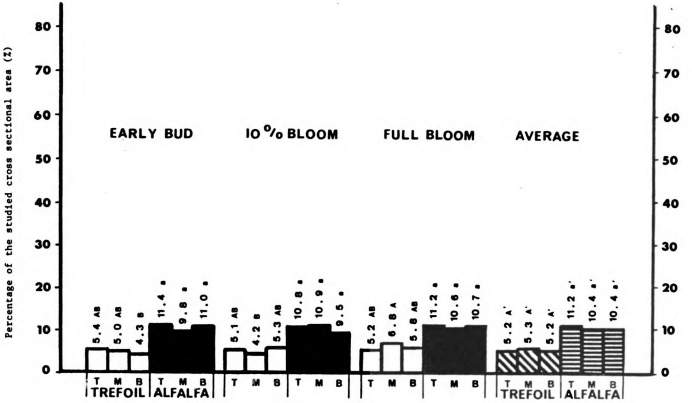
Table 13. Vertical changes of the ratio leaf cross sectional length to leaf cross sectional width of both alfalfa and birdsfoot trefoil at three maturity stages.

Species	Layers	Maturity Stages			
		Early Bud	10% Bloom	Full Bloom	Average
Alfalfa	Top (4th node)	22.3 de	22.6 de	18.9 e	21.3 C
	Middle (9th node)	31.3 bc	36.1 b	28.2 cd	31.9 B
	Bottom (15th node)	34.6 bc	34.4 bc	48.5 a	39.2 A
	Average	29.4 A	31.0 A	31.9 A	30.8 **
Trefoil	Top (4th node)	17.9 ab	15.6 ab	10.3 b	14.6 A
	Middle (9th node)	16.5 ab	21.8 a	15.0 ab	17.8 A
	Bottom (15th node)	12.6 b	17.8 ab	18.2 ab	16.2 A
	Average	15.7 A	18.4 A	14.5 A	16.2 **
Average		22.5 A	24.7 A	23.2 A	

Comparisons between layers and maturity stages within each species are shown by small letters and the means with the same letter are not different at the 5 percent level by the Duncan's Multiple Range test.

Comparisons between maturity stages and between layers within each species are shown by capital letter and the means with the same letter are not different at the 5 percent level by the Duncan's test.

Comparisons between both species (average) are done by the t-test and the means that shown difference at the 1 percent level are followed by ** .

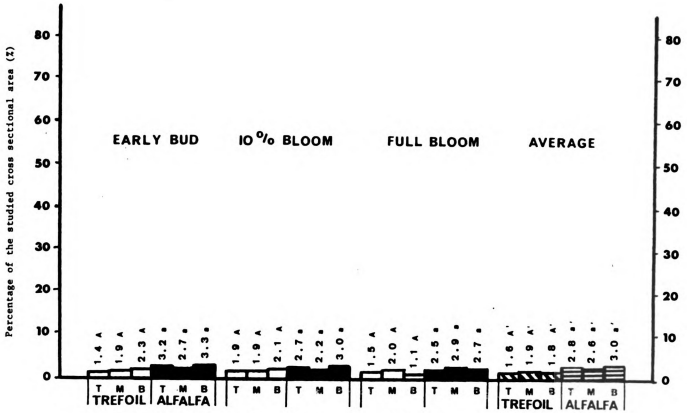


Vertical layers T: top (4^{th} node) M: middle (9^{th} node) B: bottom (15^{th} node)

Figure 7. A vertical distribution of vascular bundle in leaves of alfalfa and birdsfoot trefoil at various maturity stages.

Comparisons between layers and maturity stages are shown by capital letter within trefoil and by small letter within alfalfa and the means with the same letter are not different at the 5 percent level by the Duncan's Multiple Range test.

Comparisons between layers of the bulk data within each species are shown by capital letter with ' for trefoil and by small letters with ' for alfalfa.

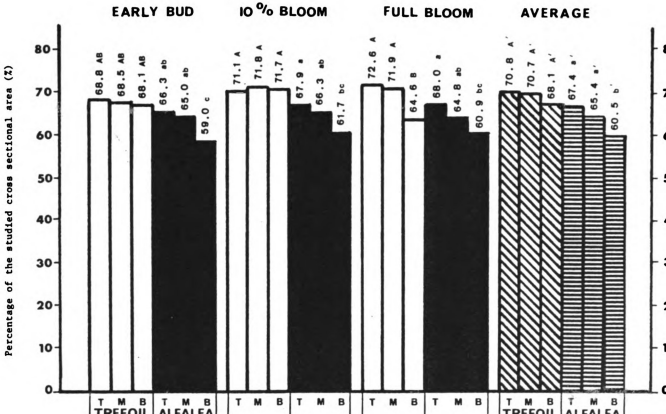


Vertical layers T: top (4th node) M: middle (9th node) B: bottom (15th node)

Figure 8. A vertical distribution of collenchyma in leaves of alfalfa and birdsfoot trefoil at various maturity stages.

Comparisons between layers and maturity stages are shown by capital letter within trefoil and by small letter within alfalfa and the means with the same letter are not different at the 5 percent level by the Duncan's Multiple Range test.

Comparisons between layers of the bulk data within each species are shown by capital letter with ' for trefoil and by small letters with ' for alfalfa.



Vertical layers T: top (4th node) M: middle (9th node) B: bottom (15th node)

Figure 9. A vertical distribution of mesophyll cells in leaves of alfalfa and birdsfoot trefoil at various maturity stages.

Comparisons between layers and maturity stages are shown by capital letter within trefoil and by small letter within alfalfa and the means with the same letter are not different at the 5 percent level by the Duncan's Multiple Range test.

Comparisons between layers of the bulk data within each species are shown by capital letter with ' for trefoil and by small letters with ' for alfalfa.

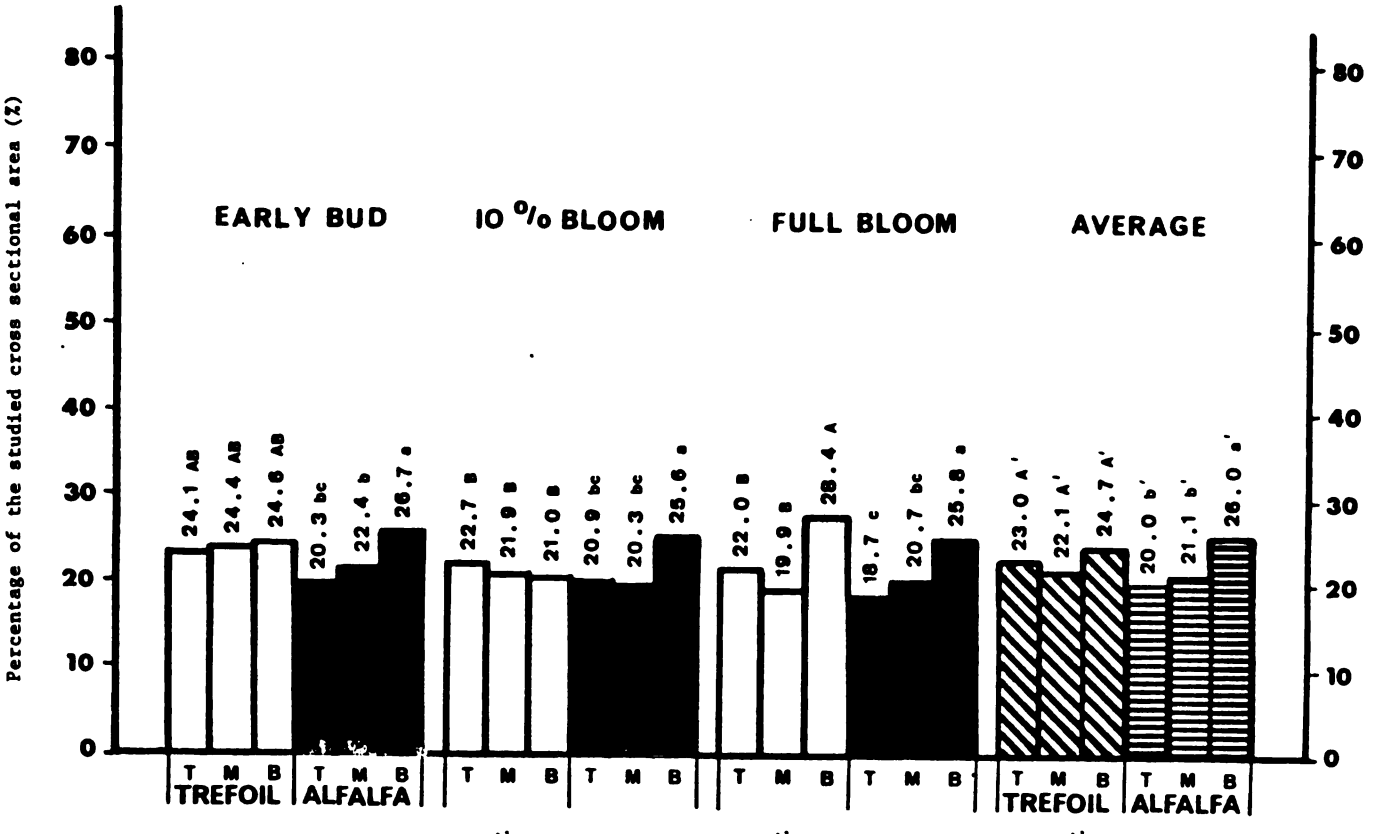
Since the leaf length of alfalfa was twice that of trefoil, the epidermal cells of the trefoil were necessarily larger than those of the alfalfa so as to show similarity in the percentage of epidermis area. Lees et al (1982) also reported larger epidermal cells in trefoil leaves than on alfalfa.

Leaves of both species did not show significant change in the amount of most tissue types vertically down the plant, except with the epidermis and the mesophyll cells (Fig. 9 and 10). No supporting results on legumes could be found in the present state of literature but Akin et al (1977) found little variation in the amount of tissue types in leaves from the upper to the basal portion of coastal bermudagrass.

The epidermal area averaged for both species ranged from 21.5 to 25.3% from plant top to bottom (Table 14). The alfalfa leaf, at every maturity stage, showed an increase of its percentage of epidermis area vertically down the plant but this increase occurred only in trefoil in full bloom (Fig. 10).

The percentage of epidermal area along the shoot of both legumes was negatively associated with the leaf thickness. On alfalfa, the correlation coefficient between the percentage of epidermis area and the leaf thickness was low $r = -0.65$ at $p < 0.01$ while on trefoil the corresponding association was $r = -0.45$ at $p < 0.05$.

The percentage of mesophyll cells in the leaf declined down the shoot of full bloom of both species as well as of alfalfa at every growth stage (Fig. 9). The percentage of mesophyll cells area was positively correlated ($p < 0.01$) with the thickness and the percentage



Vertical layers T: top (4th node) M: middle (9th node) B: bottom (15th node)

Figure 10. A vertical distribution of epidermis in leaves of alfalfa and birdsfoot trefoil at various maturity stages.

Comparisons between layers and maturity stages are shown by capital letter within trefoil and by small letter within alfalfa and the means with the same letter are not different at the 5 percent level by the Duncan's Multiple Range test.

Comparisons between layers of the bulk data within each species are shown by capital letter with ' for trefoil and by small letters with ' for alfalfa.

Table 14. Vertical changes of the mean area of anatomical components in leaf and in stem of both alfalfa and birdsfoot trefoil.

Tissue Types	Vertical Layers		
	Top (%)	Middle (%)	Bottom (%)
	Leaf		
Epidermis	21.5 b	21.6 b	25.3 a
Mesophyll cells	69.1 a	68.0 a	64.3 b
Collenchyma	2.2 a	2.2 a	2.4 a
Vascular bundle	8.2 a	7.8 a	7.7 a
Sclerenchyma	2.1 a	2.0 a	1.5 a
Phloem	2.0 a	1.8 a	1.9 a
Xylem	3.9 a	4.1 a	4.3 a
	Stem		
Epidermis	6.2 a	5.3 b	5.0 c
Chlorenchyma	16.7 a	14.6 b	14.1 b
Collenchyma	2.5 a	1.8 b	1.6 b
Bundle sheath	3.4 a	2.9 b	2.5 b
Vascular bundle	33.0 b	43.4 a	43.4 a
Sclerenchyma	9.6 b	11.2 a	11.5 a
Phloem	7.6 a	6.5 b	7.1 ab
Xylem	14.3 b	22.4 a	24.8 a
Xylem in parenchyma	3.8 a	4.1 a	3.5 a
Xylem interfascicular	12.9 b	18.0 a	16.0 a
Pith parenchyma	21.5 a	12.8 b	11.7 b

Comparisons between layers for the bulk data of both species within each tissue type are shown by small letters and the means with the same letter are not significantly different at the 5 percent level by the Duncan's test.

area of mesophyll cells ($r = +0.74$ for alfalfa and $r = +0.47$ for trefoil) (Table 15). This confirms observations of Cooper and Qualls (1967) and Pearce and Lee (1969) who found that sun leaves of both species were thicker than shade leaves and that sun leaves had more and larger palisade and mesophyll cells.

Association of leaf anatomical components with nutritive value of leaf

The anatomical components of the leaf of both legumes were not consistently related to IVDMD determined after 24 hours. However, some tissue types such as epidermis, sclerenchyma and mesophyll cells showed close relationships to the RFV (Table 16). The RFV of trefoil leaves was correlated positively with the percentages of epidermis ($r = +0.61$, $p < 0.01$) and negatively with sclerenchyma ($r = -0.42$, $p < 0.05$), and mesophyll cells ($r = -0.50$, $p < 0.01$). The leaf RFV of alfalfa leaves was positively correlated ($p < 0.01$) with the epidermis ($r = +0.62$) and negatively correlated with the mesophyll cells ($r = -0.62$).

The above results suggest that the percentage of epidermis area in leaves of both species could be used as a potential indicator of the leaf RFV. The positive correlation ($p < 0.01$) between the amount of epidermis and vascular bundles ($r = +0.55$ on trefoil and $r = +0.64$ on alfalfa) suggests that the epidermis reflected the internal anatomy of the leaf and, consequently, the intake of digestible energy.

The negative correlation between the leaf RFV and the percentage of mesophyll cells could result in a lack of structural strength of this tissue type. Consequently, the leaf intake seemed to decrease as

Table 15. Vertical changes of the leaf thickness (leaf cross sectional width) of both alfalfa and birdsfoot trefoil at three maturuty stages.

Species	Layers	Maturity Stages			
		Early Bud	10% Bloom	Full Bloom	Average
		(cm)	(cm)	(cm)	(cm)
Alfalfa	Top (4th node)	0.135 ab	0.124 bc	0.142 a	0.134 A
	Middle (9th node)	0.115 cd	0.120 bc	0.122 bc	0.117 B
	Bottom (15th node)	0.091 e	0.091 e	0.100 de	0.095 C
	Average	0.113 A	0.112 A	0.121 A	0.115 **
Trefoil	Top (4th node)	0.150 abc	0.148 abc	0.188 a	0.162 A
	Middle (9th node)	0.144 bc	0.148 abc	0.169 ab	0.154 A
	Bottom (15th node)	0.128 bc	0.153 abc	0.115 c	0.133 B
	Average	0.141 A	0.150 A	0.157 A	0.150 **
Average		0.127 A	0.131 A	0.139 A	

Comparisons between layers and maturity stages within each species are shown by small letters and the means with the same letter are not different at the 5 percent level by the Duncan's Multiple Range test.

Comparisons between maturity stages and between layers within each species are shown by capital letter and the means with the same letter are not different at the 5 percent level by the Duncan's test.

Comparisons between both species (average) are done by the t-test and the means that shown difference at the 1 percent level are followed by ** .

Table 16.Correlation coefficient between the various anatomical components in leaf of each leguminous species and their respective leaf IVDMD (24 hours) and R.F.V.

	Species			
	Trefoil		Alfalfa	
Tissue Types	IVDMD	R.F.V.	IVDMD	R.F.V.
Epidermis	+ 0.08	+ 0.61 **	+ 0.01	+ 0.62 **
Mesophyll cells	+ 0.01	- 0.50 **	- 0.11	- 0.62 **
Collenchyma	+ 0.19	- 0.21	+ 0.27	+ 0.20
Vascular bundle	- 0.04	- 0.10	+ 0.11	+ 0.23
Sclerenchyma	- 0.19	- 0.42 *	+ 0.07	- 0.02
Phloem	+ 0.02	- 0.05	+ 0.17	+ 0.22
Xylem	+ 0.03	+ 0.09	- 0.05	+ 0.25

* Correlation significantly different at the 5 percent level. ** Correlation significantly different at the 1 percent level.

the proportion of mesophyll cells in the leaf increased due to the ease of this material to be degraded and assimilated.

STEMS

Effect of species, maturity and vertical layers on anatomical components

Several anatomical components of the cross section of the stem were statistically affected by the species. There was, however, no interaction between species and maturity stages and/or plant layers that significantly affected the percentage of the tissue types (Table 17).

The percentage area of the epidermis, cortical parenchyma, interfascicular xylem, sclerenchyma and collenchyma were mainly affected by the species. In general, the stem of birdsfoot trefoil had relatively more epidermis, cortical parenchyma, sclerenchyma and interfascicular xylem than the stem of alfalfa (Table 17). In contrast, the area of collenchyma in a stem was higher in alfalfa (3.3%) than in trefoil (0.7%).

The larger cortical zone in trefoil stems compared to those in alfalfa may be due to the smallness stem diameter of trefoil (Table 10) or, less likely, as a need for a site for photosynthetic activity. Firstly, the smaller diameter of the trefoil than the alfalfa stem could have allowed a larger proportion of its cortical zone to be taken on the photomicrograph. A similar explanation could be apply to the larger amount of epidermis and interfascicular xylem in the trefoil than in the alfalfa stem. Secondly, the large amount of cortical parenchyma in the trefoil stem may be the site of some photosynthetic activity so as to compensate the lack of leafiness of trefoil as compared to that on alfalfa (Chapter I) under the present experimental conditions.

Table 17. Mean area of anatomical components in stems of alfalfa and birdsfoot trefoil and analysis of variance of the main factors: species, maturity stages and plant layers on the percentage area of anatomical components (in plant with 18 nodes development).

Tissue Types	Species		Factors		
	Alfalfa	Trefoil	S	M	P
	(%)	(%)			
Epidermis	5.0 **	6.0 **	**	N.S.	*
Chlorenchyma	12.2 **	18.1 **	**	N.S.	N.S.
Collenchyma	3.3 **	0.7 **	**	N.S.	N.S.
Bundle sheath	3.0	2.9	N.S.	N.S.	**
Vascular bundle	44.6	40.1	N.S.	N.S.	**
Sclerenchyma	9.5 **	12.3 **	*	N.S.	N.S.
Phloem	7.0	7.1	N.S.	N.S.	N.S.
Xylem	23.3	17.7	N.S.	N.S.	**
Xylem parenchyma	4.8	3.0	N.S.	N.S.	N.S.
Xylem interfascicular	13.9 **	17.9 **	**	N.S.	*
Pith parenchyma cells	18.1	13.6	N.S.	N.S.	**

The significance of factors was evaluated at 1 percent level and followed by ** and by * at 5 percent level and N.S. followed those which are not significant.

Alfalfa and trefoil differed in their vascularization development of the stem. The ratio of xylem:phloem was 4:1 for alfalfa and 3:1 for trefoil. The above characteristics of the vascular system of the trefoil stem would suggest a better adaptation of trefoil to dry periods than alfalfa. Thus, Nelson and Smith (1968) reported that trefoil displayed a steady development during a dry period while alfalfa showed a decrease of development during a dry period while alfalfa showed a decrease of development of new organs in similar conditions. However, some defects of trefoil, such as its slow growth and its susceptibility to lodge, could also be associated to its vascular system.

Trefoil favors sclerenchyma for strenghtning the stem tissue in the cortex. The ratio of the amount of sclerenchyma to the amount of collenchyma in the stem averaged 12:1 in trefoil and 3:1 in alfalfa. In the alfalfa stem, the sclerenchyma were around the vascular vessels (separated of the collenchyma by the bundle sheath).

The need of mechanical tissues was particularly important for alfalfa which had a larger stem as well as many vascular bundles. Thus, alfalfa stems averaged 0.133 cm in diameter and 16 vascular bundles while the trefoil stem had a mean diameter of 0.110 cm and nine vascular bundles (Table 18). For both legumes, the number of vascular bundles was positively correlated ($p < 0.01$) to the amount of sclerenchyma $r = +0.61$ and to the amount of collenchyma $r = +0.71$.

Changes in stem tissue types in vertical layers and according to maturity

The percentage of the epidermis, bundle sheath, interfascicular xylem, xylem, vascular bundle and parenchyma cells in the pith was sta-

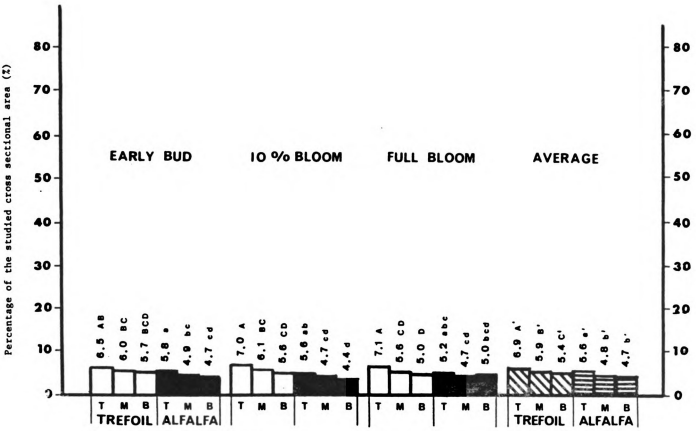
tiscally affected by the stem position on the shoot but not by maturity (Table 17).

Epidermis

The mean percentage of epidermis area of the legume at both stages of maturity decreased from 6.2 to 5.0% down the shoot (Table 14). Stem diameter (Table 10) and the percentage of epidermis area ($r = -0.75$) and the number of vascular bundles and the percentage of epidermis area ($r = -0.77$) were negatively correlated ($p < 0.01$). It could then be hypothesized that the epidermal layer would become more elongated with thicker walls for strenthening the tissues as the internal structure of the stem increased in size. Reciprocally, the decrease of the epidermis area in the stem could result in its declining importance down the shoot compared to the area of some other tissue types such as vascular bundles. For instance, a significant negative association was found between the epidermis area and the vascular bundle area ($r = -0.78$, $p < 0.01$).

The mean percentage of epidermis area in alfalfa declined from the upper (5.6%) to the median (4.8%) and basal stem (4.7%) (Fig. 11). The epidermis in trefoil gradually declined from the top (6.9%) to bottom (5.4%). The difference could be due to the variation in the influence that exerted the number of vascular bundles (Table 18) on the amount of epidermis. Thus, there was a significant and negative association of $r = -0.53$, $p < 0.01$ between the percentage of epidermis area and the number of vascular bundles on trefoil but not in alfalfa.

The percentage of epidermis area along the shoot was constant in early bud trefoil and full bloom alfalfa, suggesting that both spe-



Vertical layers T: top (4^{th} node) M: middle (9^{th} node) B: bottom (15^{th} node)

Figure 11. A vertical distribution of epidermis in stems of alfalfa and birdsfoot trefoil at various maturity stages.

Comparisons between layers and maturity stages are shown by capital letter within trefoil and by small letter within alfalfa and the means with the same letter are not different at the 5 percent level by the Duncan's Multiple Range test.

Comparisons between layers of the bulk data within each species are shown by capital letter with ' for trefoil and by small letters with ' for alfalfa.

Table 18. Vertical distribution of the number of vascular bundles in stem cross section of alfalfa and birdsfoot trefoil at three maturity stages.

Species	Layers	Maturity Stages			
		Early Bud	10 % Bloom	Full Bloom	Average
Alfalfa	Top (4th node)	15 ab	14 b	14 b	14 B
	Middle (9th node)	16 ab	16 ab	15 ab	16 AB
	Bottom (15th node)	17 ab	18 a	16 ab	17 A
	Average	16 A	16 A	15 A	16 **
Trefoil	Top (4th node)	9 cde	8 e	8 de	8 B
	Middle (9th node)	10 ab	9 bcd	10 abc	10 A
	Bottom (15th node)	11 a	10 abc	10 ab	10 A
	Average	10 A	9 A	9 A	9 **
Average		13 A	12 A	12 A	—

Comparisons between layers and maturity stages within each species are shown by small letters and the means with the same letter are not different at the 5 percent level by the Duncan's Multiple Range test.

Comparisons between maturity stages and between layers within each species are shown by capital letter and the means with the same letter are not different at the 5 percent level by the Duncan's test.

Comparisons between both species (average) are done by the t-test and the means that shown difference at the 1 percent level are followed by ** .

cies may have either a differential process of aging of the shoot apical meristem or a differential response of the stem anatomy upon the shoot growth rate.

Bundle sheath, parenchyma cells

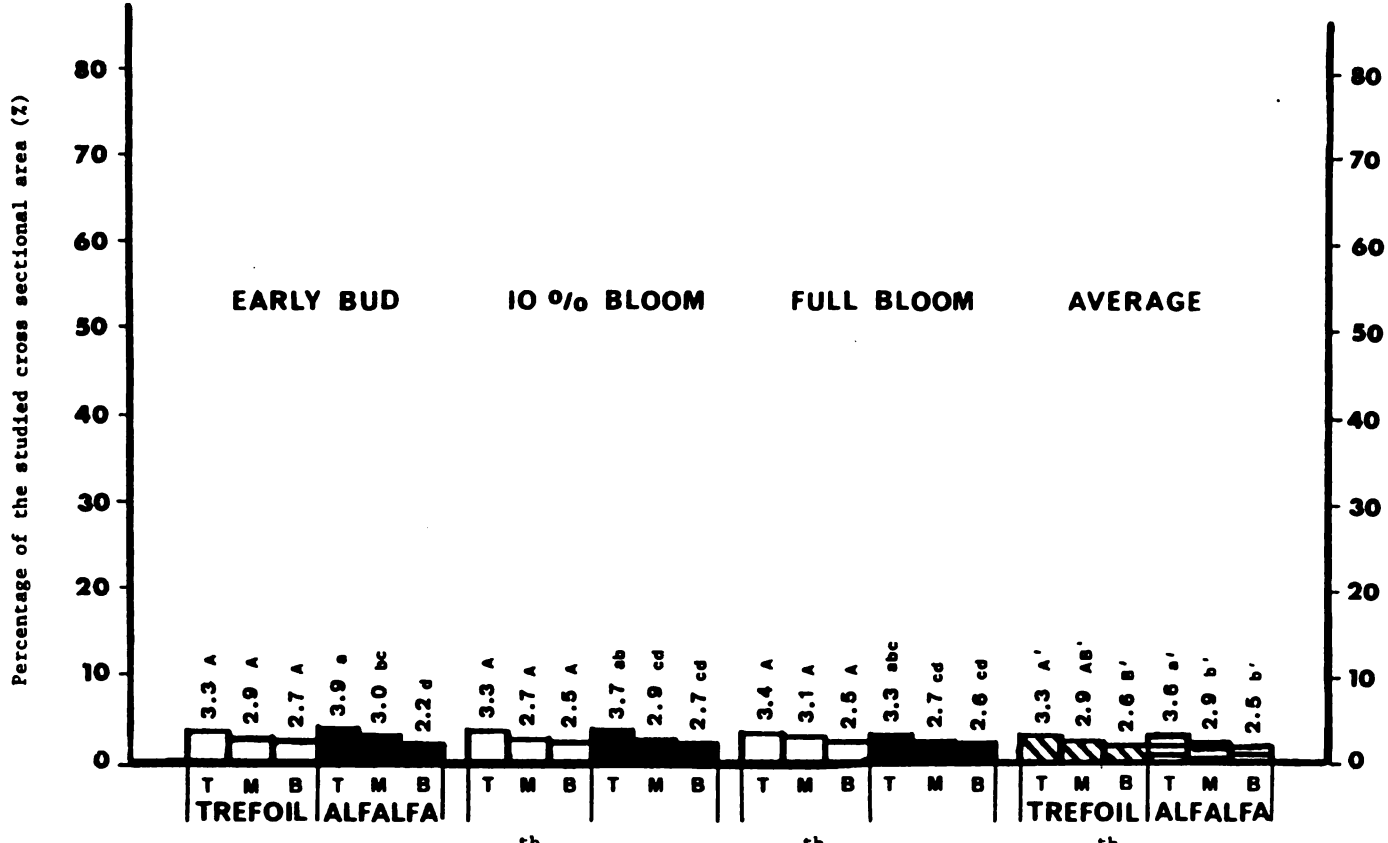
The combined data of both legumes showed that area of the bundle sheath and pith parenchyma cells area of the stem declined from top to middle and, then remained, unchanged (Table 14). These results could be related to the size of the vascular bundle which was influenced by the shoot age. As the vascular bundle became larger, the area of bundle sheath as well as of parenchyma cells in the pith became smaller (Fig. 12, 13). The area percentage of the bundle sheath was negatively correlated ($p < 0.05$) with the vascular bundle ($r = -0.67$) and with the parenchyma cells in the pith and the vascular bundle area ($r = -0.68$).

Interfascicular xylem, xylem and vascular bundles

The percentage area of interfascicular xylem, xylem and vascular bundles of each legume increased from top to middle and then remained stable toward the basal stem portion (Fig. 14, 15, 16), particularly for the xylem (Fig. 18). The percentage of the xylem and vascular bundle area seemed to be related to the rate of increase of the stem diameter.

Association of the stem components with plant nutritive value

The percentage of the cross sectional area of stem tissue types was related to their stem IVDMD. The percentage area of parenchyma cells in the pith, bundle sheath and epidermis were correlated with the stem IVDMD in both alfalfa and trefoil (Table 19). The chlorenchyma, collenchyma and phloem of alfalfa were highly correlated with the IVDMD of the stem.

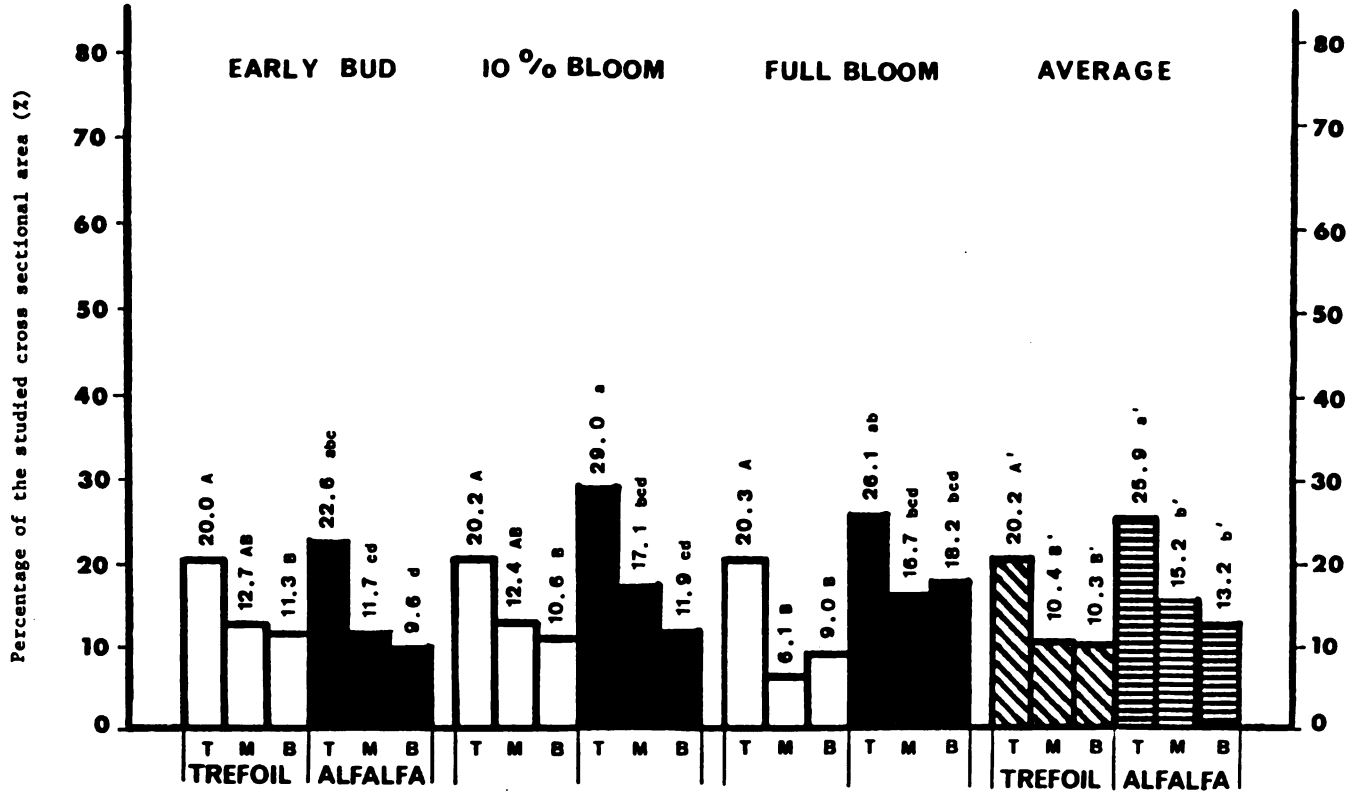


Vertical layers T: top (4^{th} node) M: middle (9^{th} node) B: bottom (15^{th} node)

Figure 12. A vertical distribution of bundle sheath in stems of alfalfa and birdsfoot trefoil at various maturity stages.

Comparisons between layers and maturity stages are shown by capital letter within trefoil and by small letter within alfalfa and the means with the same letter are not different at the 5 percent level by the Duncan's Multiple Range test.

Comparisons between layers of the bulk data within each species are shown by capital letter with ' for trefoil and by small letters with ' for alfalfa.

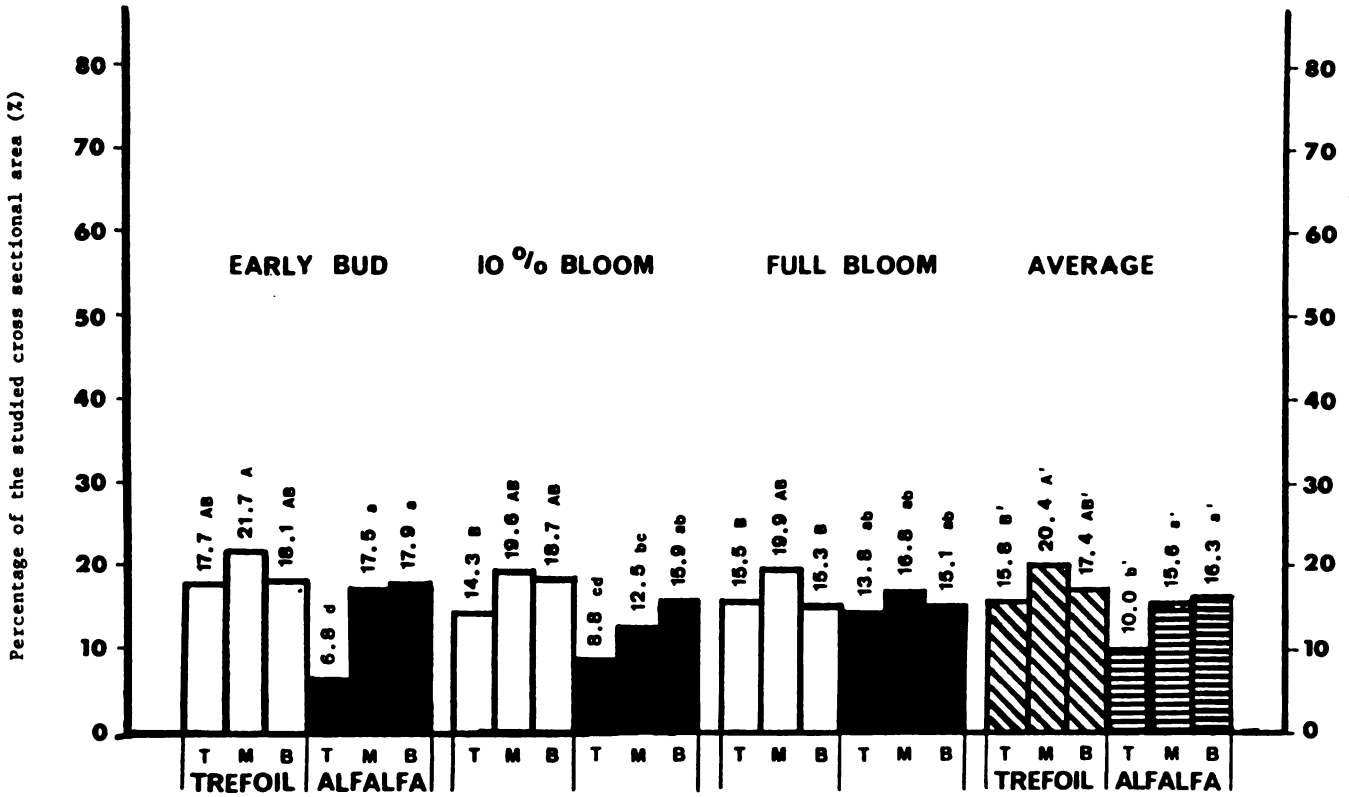


Vertical layers T: top (4^{th} node) M: middle (9^{th} node) B: bottom (15^{th} node)

Figure 13. . A vertical distribution of parenchyma cells in the pith of stems of alfalfa and birdsfoot trefoil at various maturity stages.

Comparisons between layers and maturity stages are shown by capital letter within trefoil and by small letter within alfalfa and the means with the same letter are not different at the 5 percent level by the Duncan's Multiple Range test.

Comparisons between layers of the bulk data within each species are shown by capital letter with ' for trefoil and by small letters with ' for alfalfa.

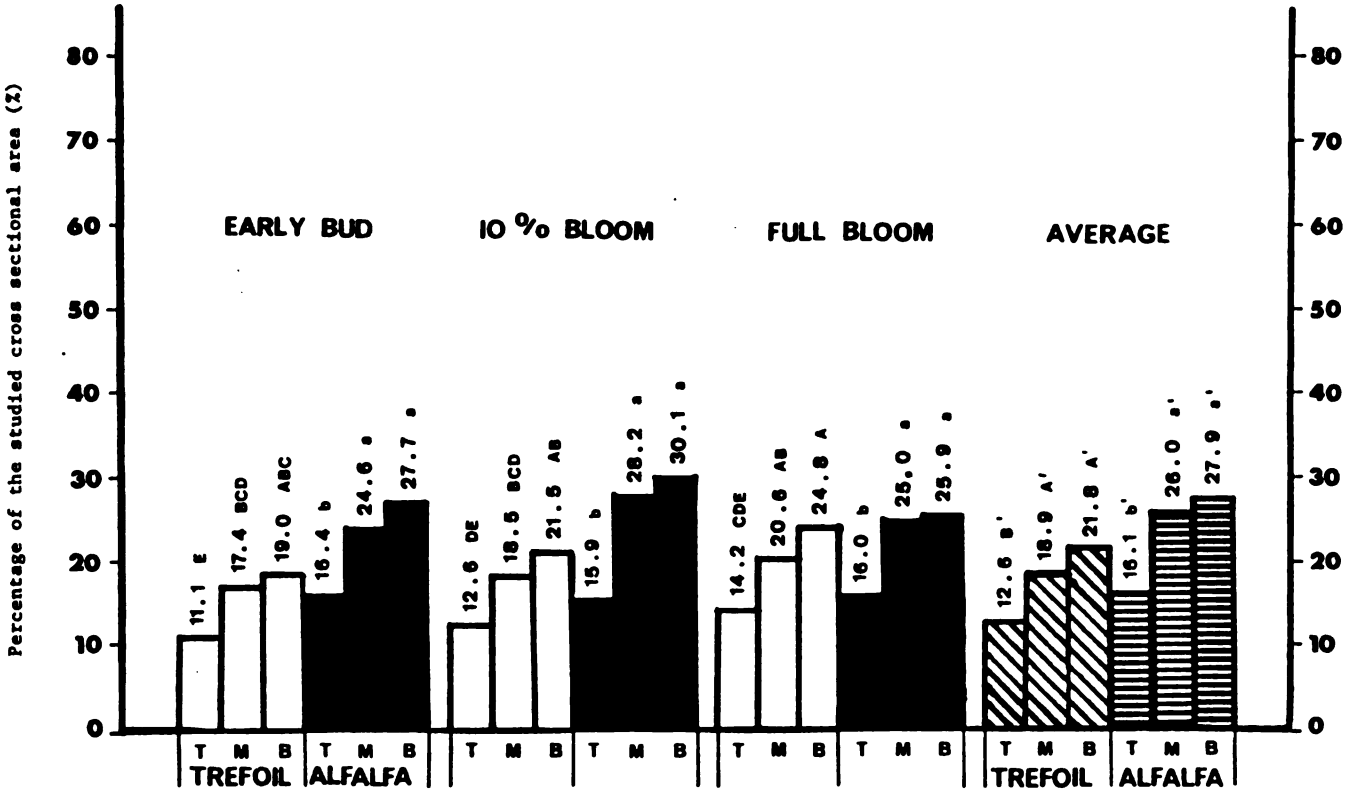


Vertical layers T: top (4th node) M: middle (9th node) B: bottom (15th node)

Figure 14. A vertical distribution of interfascicular xylem in stems of alfalfa and birdsfoot trefoil at various maturity stages.

Comparisons between layers and maturity stages are shown by capital letter within trefoil and by small letter within alfalfa and the means with the same letter are not different at the 5 percent level by the Duncan's Multiple Range test.

Comparisons between layers of the bulk data within each species are shown by capital letter with ' for trefoil and by small letters with ' for alfalfa.

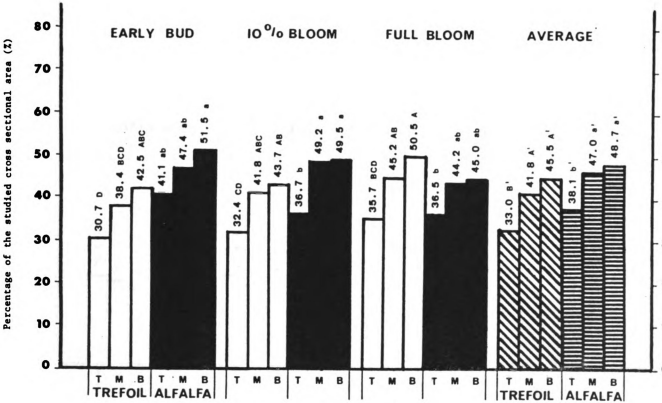


Vertical layers T: top (4^{th} node) M: middle (9^{th} node) B: bottom (15^{th} node)

Figure 15. A vertical distribution of xylem in stems of alfalfa and birdsfoot trefoil at various maturity stages.

Comparisons between layers and maturity stages are shown by capital letter within trefoil and by small letter within alfalfa and the means with the same letter are not different at the 5 percent level by the Duncan's Multiple Range test.

Comparisons between layers of the bulk data within each species are shown by capital letter with ' for trefoil and by small letters with ' for alfalfa.

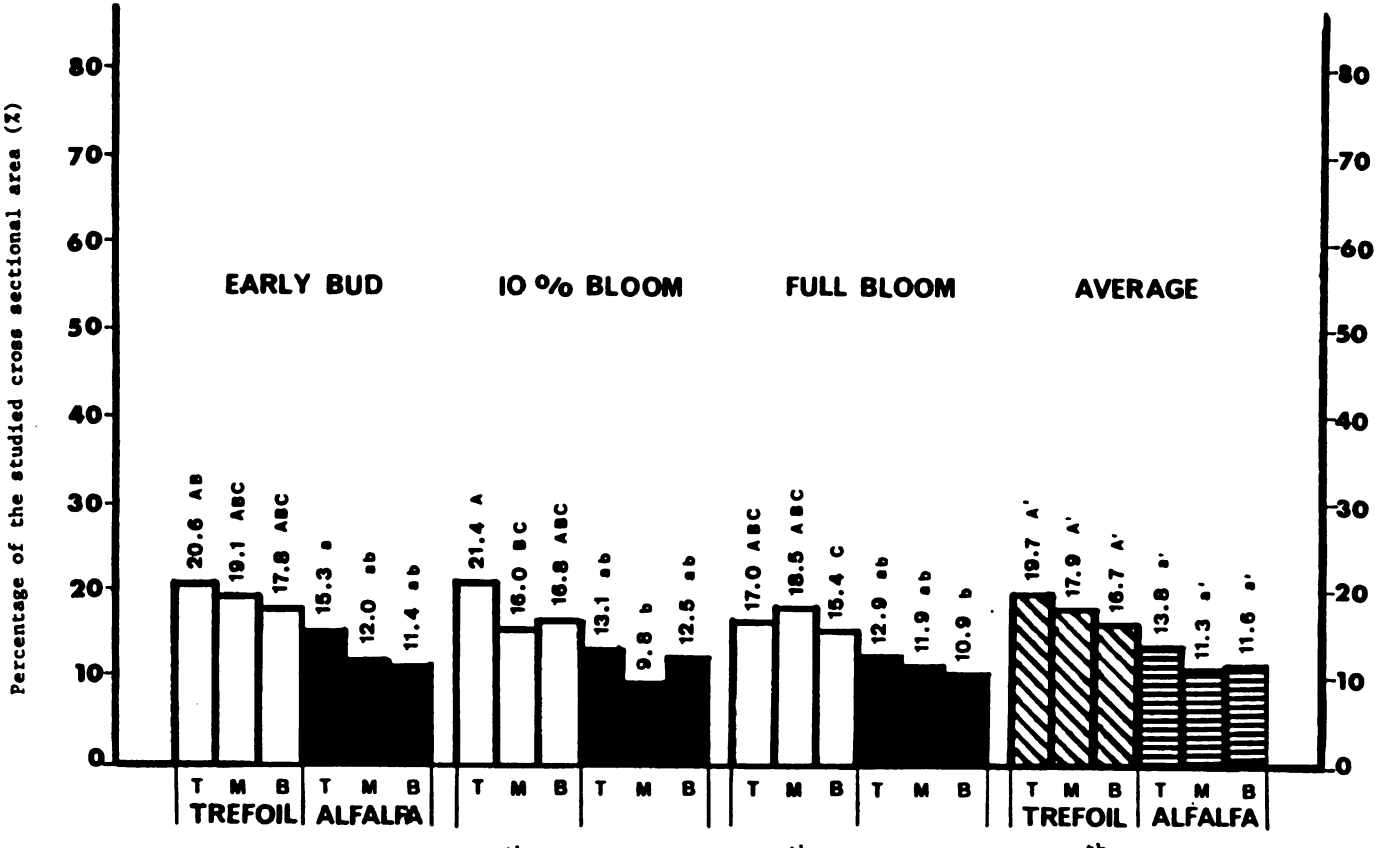


Vertical layers T: top (4^{th} node) M: middle (9^{th} node) B: bottom (15^{th} node)

Figure 16. A vertical distribution of vascular bundle in stems of alfalfa and birdsfoot trefoil at various maturity stages.

Comparisons between layers and maturity stages are shown by capital letter within trefoil and by small letter within alfalfa and the means with the same letter are not different at the 5 percent level by the Duncan's Multiple Range test.

Comparisons between layers of the bulk data within each species are shown by capital letter with ' for trefoil and by small letters with ' for alfalfa.

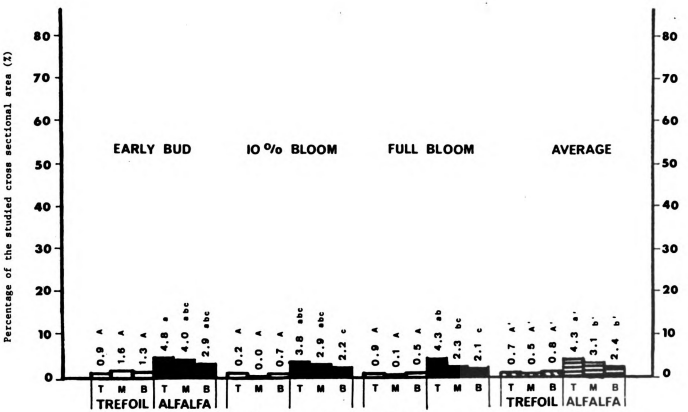


Vertical layers T: top (4th node) M: middle (9th node) B: bottom (15th node)

Figure 17. A vertical distribution of chlorenchyma in stems of alfalfa and birdsfoot trefoil at various maturity stages.

Comparisons between layers and maturity stages are shown by capital letter within trefoil and by small letter within alfalfa and the means with the same letter are not different at the 5 percent level by the Duncan's Multiple Range test.

Comparisons between layers of the bulk data within each species are shown by capital letter with ' for trefoil and by small letters with ' for alfalfa.



Vertical layers T: top (4th node) M: middle (9th node) B: bottom (15th node)

Figure 18. A vertical distribution of collenchyma in stems of alfalfa and birdsfoot trefoil at various maturity stages.

Comparisons between layers and maturity stages are shown by capital letter within trefoil and by small letter within alfalfa and the means with the same letter are not different at the 5 percent level by the Duncan's Multiple Range test.

Comparisons between layers of the bulk data within each species are shown by capital letter with ' for trefoil and by small letters with ' for alfalfa.

Table 19. Correlation coefficient between the various anatomical components in stem of each legume and their respective stem IVDMD (24 hours) and RFV.

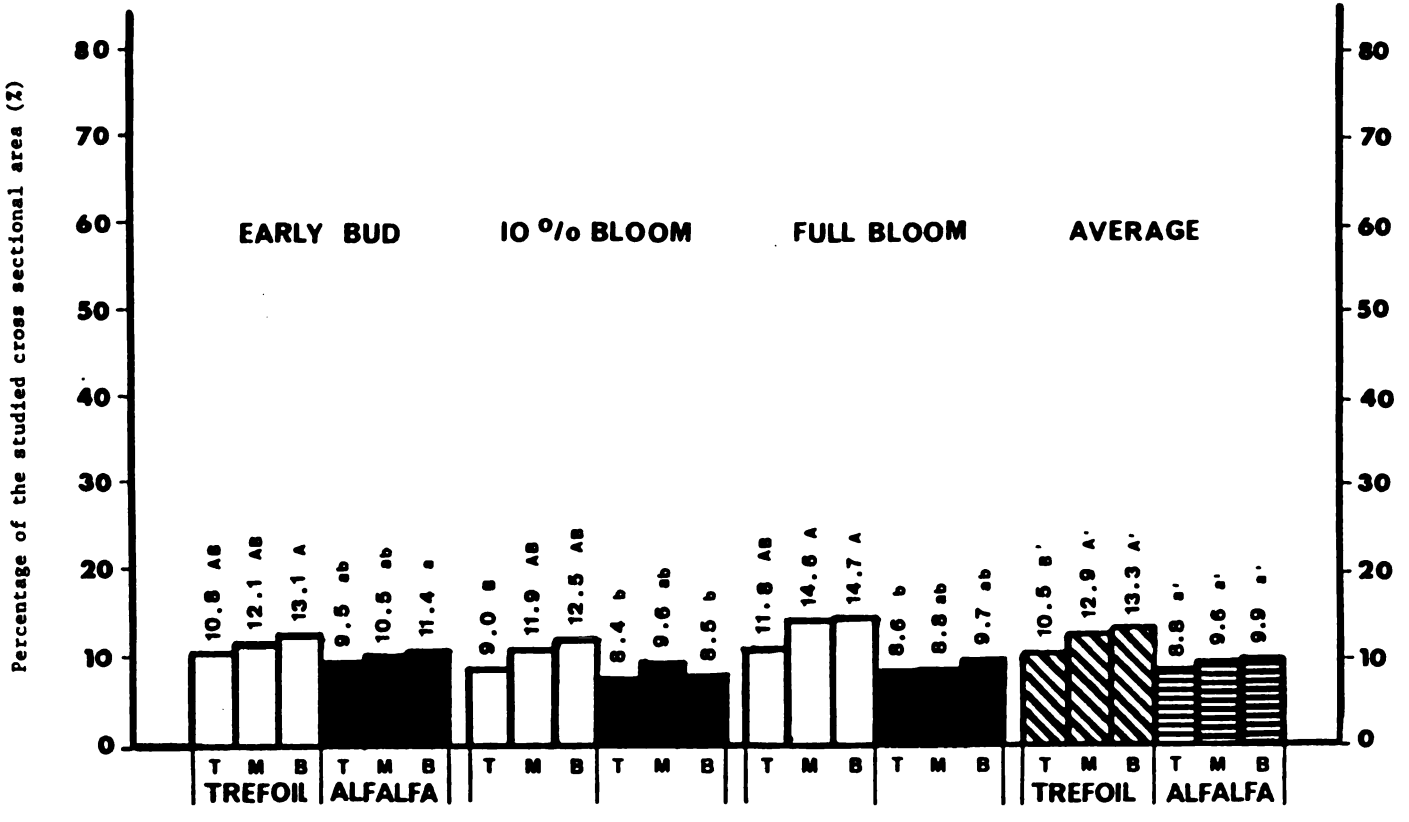
Tissue Types	Species			
	Trefoil		Alfalfa	
	IVDMD	RFV	IVDMD	RFV
Epidermis	+ 0.63 **	+ 0.74 **	+ 0.38 *	+ 0.41 *
Chlorenchyma	+ 0.30	+ 0.19	+ 0.37 *	+ 0.41 *
Collenchyma	+ 0.02	+ 0.07	+ 0.51 **	+ 0.46 *
Bundle sheath	+ 0.41 *	+ 0.46 *	+ 0.61 **	+ 0.64 **
Vascular bundle	- 0.61 **	- 0.66 **	- 0.47 *	- 0.56 **
Sclerenchyma	- 0.36 *	- 0.44 *	- 0.22	- 0.34
Phloem	- 0.24	- 0.20	+ 0.43 *	+ 0.41 *
Xylem	- 0.63 **	- 0.70 **	- 0.67 **	- 0.69 **
Parenchyma in xylem	- 0.13	- 0.14	+ 0.25	+ 0.14
Interfascicular xylem	- 0.04	- 0.12	- 0.53 **	- 0.54 **
Pith parenchyma	+ 0.55 **	+ 0.71 **	+ 0.50 **	+ 0.53 **

* Correlation significantly different at the 5 percent level ** Correlation significantly different at the 1 percent level.

In contrast, the trefoil stem displayed a negative and significant relationship between the percentage of cross sectional area of xylem, sclerenchyma, vascular bundle, and the stem IVDMD. In alfalfa, a negative correlation occurred between the percentage of cross sectional area of vascular bundle, interfascicular xylem and the stem IVDMD. The previous data confirmed some the aspects of Shenk and Elliot (1971) who reported a negative influence of the stem percentage area of vascular bundles and xylem on the stem IVDMD of alfalfa.

Besides the influence of the stem anatomy on the stem IVDMD, the level of intake digestible energy (RFV) of stems of both legume was highly correlated with the living tissues of epidermis, pith parenchyma cells and bundle sheath which change morphologically with the plant age and had a positive influence on the RFV of stems of both species (Table 19). The RFV of alfalfa was positively correlated ($p < 0.05$) with the percentage of cross sectional area of collenchyma ($r = +0.46$), of chlorenchyma ($r = +0.41$), and of phloem ($r = +0.41$).

Conversely, the RFV of the trefoil stem was negatively correlated with the percentage of cross sectional area of supporting tissues such as vascular bundle ($r = -0.66$, $p < 0.01$), xylem ($r = -0.70$, $p < 0.01$) and sclerenchyma ($r = -0.40$, $p < 0.05$). The RFV of the alfalfa stem was correlated ($p < 0.01$) with the percentage area of vascular bundle ($r = -0.56$), of xylem ($r = -0.69$), and of interfascicular xylem ($r = -0.54$).

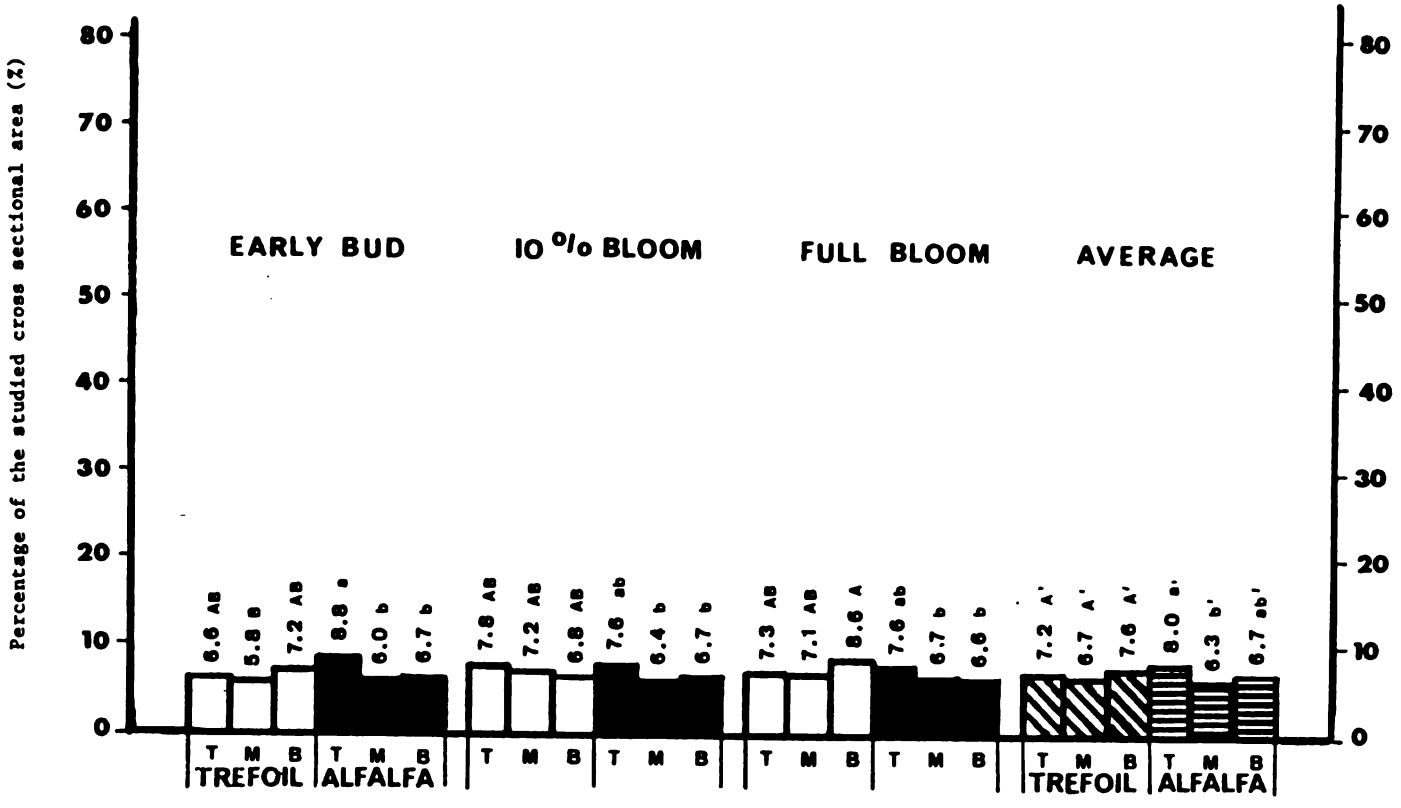


Vertical layers T: top (4^{th} node) M: middle (9^{th} node) B: bottom (15^{th} node)

Figure 19. A vertical distribution of sclerenchyma in stems of alfalfa and birdsfoot trefoil at various maturity stages.

Comparisons between layers and maturity stages are shown by capital letter within trefoil and by small letter within alfalfa and the means with the same letter are not different at the 5 percent level by the Duncan's Multiple Range test.

Comparisons between layers of the bulk data within each species are shown by capital letter with ' for trefoil and by small letters with ' for alfalfa.

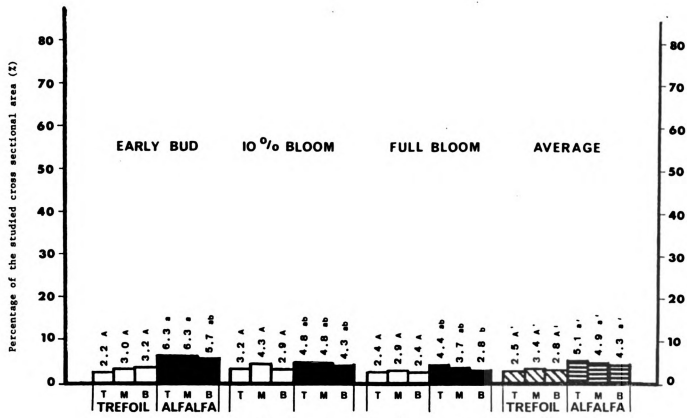


Vertical layers T: top (4th node) M: middle (9th node) B: bottom (15th node)

Figure 20. A vertical distribution of phloem in stems of alfalfa and birdsfoot trefoil at various maturity stages.

Comparisons between layers and maturity stages are shown by capital letter within trefoil and by small letter within alfalfa and the means with the same letter are not different at the 5 percent level by the Duncan's Multiple Range test.

Comparisons between layers of the bulk data within each species are shown by capital letter with ' for trefoil and by small letters with ' for alfalfa.



Vertical layers T: top (4th node) M: middle (9th node) B: bottom (15th node)

Figure 21. A vertical distribution of parenchyma cells in xylem of vascular bundles of stem of alfalfa and birdsfoot trefoil at various maturity stages.

Comparisons between layers and maturity stages are shown by capital letter within trefoil and by small letter within alfalfa and the means with the same letter are not different at the 5 percent level by the Duncan's Multiple Range test.

Comparisons between layers of the bulk data within each species are shown by capital letter with ' for trefoil and by small letters with ' for alfalfa.

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CHAPTER III

CHEMICAL COMPOSITION
OF
ALFALFA AND BIRDSFOOT TREFOIL

ABSTRACT

Alfalfa (*Medicago sativa L.*) and birdsfoot trefoil (*Lotus corniculatus L.*) were grown in two greenhouse experiments to determine 1) the changes in cell wall constituents at early bud and full bloom and 2) the vertical and horizontal changes in cell wall constituents of leaf and stem of plants with 18 nodes.

The NDF fraction, the cellular content and the wall crude protein were affected by the morphological development of the legume. Trefoil had a higher NDF content and a wall crude protein than alfalfa. The level of cell solubles was greater in alfalfa than in trefoil.

The NDF fraction increased in trefoil with age and decreased in alfalfa. The level of cellular content increased in alfalfa and decreased in trefoil with age. Trefoil increased in p-lignin from early bud to full bloom and was unchanged in alfalfa.

Morphological development affected the level of hemicelluloses and cellulose. Plants with a high number of nodes (24 to 28) had lower structural polysaccharides than plants with a node number from 10 to 22.

The mean RFV of alfalfa and trefoil did not markedly change with maturity.

The leaf and stem of trefoil had a higher level of NDF, ADF and cell wall protein than alfalfa. The levels of p-lignin and cellulose were higher in the leaf and stem of trefoil than in alfalfa. Cellulose in leaves was higher in alfalfa than in trefoil. Cellular content in alfalfa was higher than in trefoil in both leaf and stem.

With maturity, the level of cell solubles and most cell wall constituents in both leaf and stem remained unchanged, except in trefoil where leaf hemicelluloses declined from early bud to full bloom.

Basal leaves contained greater NDF, ADF and cellulose than the upper and median leaves. The RFV of leaf was greater in basal than in the upper leaves. In the stem, levels of hemicelluloses and wall protein were similar in the median and basal layer. Cell wall protein level was higher in the upper than in the two lower stem portions. The RFV of the stem declined from top to bottom.

INTRODUCTION AND LITTERATURE REVIEW

Quality of forage implies several biological, chemical and physiological characteristics. These features could have an important influence on forage utilisation, digestion and animal performance. Difference in digestibility between alfalfa and birdsfoot trefoil have been reported by Horrocks and Washko (1968) and Allinson et al (1969). However, few studies on both legumes have been reported in order to show vertical changes in content of various forage nutritional components during maturation.

According to Van Soest (1967, 1973) the nutritional components of herbage can be divided into two main categories. First, the cellular soluble protein, lipids, starch and pectin represent the maximum fraction of the forage readily and almost completely digested (Van Soest et al 1966, Barnes 1973, Moir 1982). Secondly, the cell walls are made up of hemicellulose, cellulose, pectin, lignin and bound protein (Van Soest 1967, 1975; Theander and Åman 1980). These structural components of plant cell wall constitute the partially digestible fraction (Bailey 1973, Barnes 1973, Moir 1982). The hemicellulose, cellulose and pectin are important sources of energy for ruminants (Dehority 1973, Daughtry et al 1978).

Van Soest (1963 (a), 1963 (b), 1964, 1965) developed an analytical procedure for forages widely accepted by animal nutritionists as a standard quality assay. This procedure has been selected for the present research.

The Van Soest's procedure consists of a two detergent extraction that removes soluble carbohydrates and leaves fibrous carbohydrate

residue. The cell content and pectin are removed during the first step of hydrolysis. Hemicelluloses and more pectin are hydrolysed by the acid detergent solution.

Experiments on orchardgrass, ryegrass, and fescue (Jarrige 1963) on alfalfa (Bailey et al 1971) and on birdsfoot trefoil (Lopez et al 1966) showed that the stems had higher levels of cellulose, of hemicellulose and of lignin than the leaves. A high level of these latter components give more firmness to the stem structure (Harkin 1973 and Bailey 1973).

Coughman (1960) showed alfalfa stems containing about three times more lignin than the leaves. According to Jung et al (1969) the cellulose content of the stem represent approximately 75% of the plant. The alfalfa stem contained less bound protein than the leaves (Coughman 1960).

The cellulose content in leaves of alfalfa and birdsfoot trefoil ranged from 6 to 12% while in their stems it varied from 12 to 33% (Hirst et al 1959, Lopez et al 1966). Tomlin et al (1965) found that alfalfa and birdsfoot trefoil contained, respectively, 3.5 and 5.1% of Klason lignin in leaves as compared to 6.4 and 10.0% in stems of immature plants. The level of hemicelluloses varied from 5 to 7% in the leaf to 4 to 7% in the stem of alfalfa while it varied from 7 to 11% in leaves to 24 to 28% in the stem of birdsfoot trefoil (Hirst et al 1959 and Lopez et al 1966). Variation in organic composition of plant parts may also occur inside of each species as shown by several authors on various forage species (Bhat and Christie 1975, Stobbs 1973, Wilkinson et al 1970).

Terry and Tilley (1964) and Christian et al (1970) separated the alfalfa plant into three segments. The bottom stem contained a hig-

her level of cellulose than the top stem. Conversely, the upper stem contained a higher level of nitrogen than the basal stem. The same authors found that the organic composition of top stem approched that of leaves. Sotola (1933) and Christian et al (1970) reported little change in chemical composition of the alfalfa leaf with the position on the shoot.

As the plant matured, some changes in chemical composition could occur in plant parts of forage species. Harkin (1973) reported that leaf of some legumes showed an increase of hemicellulose and lignin with increasing maturity. Wilman et al (1977) found a slight reduction in the proportion of hemicellulose in leaves during maturation. The fiber content in alfalfa leaves showed a greater uniformity than stem parts with age (Woodman and Evans 1935).

The objectives of the present study were to a) determine the changes in the cell wall constituents among various groups of alfalfa and birdsfoot trefoil collected at the same stage of maturity, b) determine the cell wall constituents of leaf and stem of both alfalfa and birdsfoot trefoil, c) determine the changes in the cell wall constituents down the shoot as well as maturity stage, d) compare the composition of cell wall of both leaf and stem of alfalfa and birdsfoot trefoil, and e) compare the cell wall constituents of plant parts between both alfalfa and trefoil.

METHODOLOGY

Plant material was grown, selected and prepared for chemical composition in the same manner as in previous sections (I, II). Extraction of plant cell wall components was carried out sequentially with neutral detergent solution (sodium lauryl sulfate) and acid detergent solution (cetyl trimethylammonium bromide) and potassium permanganate ($KMnO_4$) on ADF (Goering and Van Soest 1970, Van Soest and Robertson 1979). Hydrolysis of the NDF fraction with an acid detergent solution was extended to two hours in order to remove most of the pectic substances and hemicelluloses as suggested by Bailey and Ulyatt (1970). Both sodium sulphite and decalin were not used as they introduced errors in estimation of some structural components.

After each hydrolysis, the residues NDF, ADF, cellulose were recovered in a tared crucible (no. 1 porosity glass sinter). The NDF and ADF residues were thoroughly rinsed with hot water. The NDF and the cellulose were finally rinsed with acetone. In each case, the residues were then removed from the crucibles, ground with dry ice in a mortar so as to break the thick crust and to get a more homogenous sample in contact with the reagents. The sample was then put back in a tared crucible and the moisture released from the residues by in vacuo over phosphorus pentoxide. The dry weight of the sample was taken and the residue sample was then ready for a subsequent hydrolysis.

The chemical components recovered with the detergent system

analysis were respectively the NDF, the cellular content (100-NDF), the ADF, the hemicellulose (NDF-ADF), the lignin (permanganate on ADF) and finally the cellulose. Nitrogen in the NDF was estimated by a micro-Kjeldahl method. The percentage of protein in the cell wall was calculated as percentage nitrogen x 6.25.

The relative feeding value (RFV) of a forage is an estimate of the digestible dry matter intake (Barnes et al 1977). The NDF and ADF fractions were used to calculate RFV for legumes as follows: Digestible dry matter (DDM) = 65.5 + 0.995 ADF% - 0.0277 $ADF\%^2$; Dry matter intake (DMI) = 39 + 2.68 NDF% - 0.0410 $NDF\%^2$; Digestible dry matter intake (DDMI) = DDM x DMI/100; Relative Feeding Value (RFV) DDMI x 2.5 .

Each component of plant material analysed was expressed as percentage of the initial dry weight. An analysis of variance was performed following a 2 x 2 x 10 with two replications in experiment I. Another analysis of variance was done following this time a 2 x 2 x 3 with four replications for each plant part. The Duncan's Multiple Range test was also used to determine the difference between treatments in each species as well as in each maturity stage in experiment I while in experiment II it was between treatments in each species as well as in both maturity stages.

A t-test was performed to compare treatments between both species in a same maturity stage in both experiments. In addition, the t-test was also used to determine the difference between treatments between species.

Each component of plant material analysed was expressed as percentage of the initial dry weight.

RESULTS AND DISCUSSION

EXPERIMENT I

NDF and cellular content

The NDF fraction and the cellular content were affected by species. Most factor interactions were not significant, except that the NDF and cellular content of species were differently affected by maturity (Table 20).

Species

Trefoil had a higher NDF (44%) and a lower cellular content (56%) than alfalfa (NDF 41.6% and 58.4%; Table 21). A close association did exist between the ratio of leaf and stem of both legumes (Chapter I) and the content of NDF as well as cellular content; $r = -0.64$, $p < 0.01$ and $r = +0.64$, $p < 0.01$, respectively (Table 22). The latter relationship was possible through the one existing in trefoil with a correlation between the leaf-stem ratio and the NDF content and the cellular content respectively $r = -0.93$, $p < 0.01$ and $r = +0.93$, $p < 0.01$ (Table 23).

The increase of NDF in trefoil herbage was due to the major structural components of hemicellulose ($r = +0.57$, $p < 0.01$), cellulose ($r = +0.56$, $p < 0.05$), and lignin ($r = +0.48$, $p < 0.05$) indicating the great influence of the stem fraction in the trefoil forage. In contrast, the alfalfa forage increased its NDF level through lignification. A positive correlation existed between the alfalfa p-lignin level and the NDF content ($r = +0.48$, $p < 0.05$; Table 23).

Species x Maturity Stages

As the plant passed from early bud to full bloom, the NDF frac-

Table 20. Analysis of variance of the factors: species (S), maturity stages (M) and morphological development (D) and their interactions on the level of various feeding value variables of the whole plant with node number ranging from 10 to 28 , in experiment I.

Variables	Main Effects and Interactions						
	S	M	D	S x M	S x D	M x D	S x M x D
NDF	**	N.S.	N.S.	**	N.S.	N.S.	N.S.
Cellular contents	**	N.S.	N.S.	**	N.S.	N.S.	N.S.
ADF	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
Hemicelluloses	N.S.	N.S.	**	N.S.	N.S.	N.S.	N.S.
p-Lignin	N.S.	*	N.S.	N.S.	N.S.	N.S.	N.S.
Celluloses	N.S.	N.S.	*	N.S.	N.S.	N.S.	N.S.
Wall crude protein	**	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
R.F.V.	N.S.	N.S.	N.S.	*	N.S.	N.S.	N.S.

The significance of factors was evaluated at 1 percent level and followed by ** and by * at 5 percent level and N.S. followed those which are not significant.

Table 21. RFV and mean level of feeding variables in forage of both alfalfa and birdsfoot trefoil (Exp. I).

	Species	
	Alfalfa	Trefoil
NDF	41.6 *	44.0 *
Cellular content	58.4 *	56.0 *
ADF	30.1	31.2
Hemicelluloses	11.7	12.0
p-Lignin	8.0	8.6
Cellulose	18.0	17.5
Wall crude protein	2.39 *	3.21 *
R.F.V.	141.1	135.6

Comparisons between both species within each variable result of t-test and the means that showed difference at 5 percent level are followed by * .

Table 22. Correlation coefficient between the leaf-stem ratio and the feeding variables as well as among the cell wall constituents of the bulk data of both studied legumes, in experiment I.

Species	Variables	Leaf-stem ratio	(1)	(2)	(3)	(4)	(5)	(6)	(7)
Alfalfa-trefoil	NDF (1)	-0.64 **							
	Cellular content (2)	+0.64 **	-1.00**						
	ADF (3)	-0.46 **	+0.78**	-0.78**					
	Hemicelluloses (4)	-0.47 **	+0.49**	-0.49**	+0.10				
	p-Lignin (5)	-0.33 *	+0.57**	-0.57**	+0.65**	-0.07			
	Cellulose (6)	-0.34 *	+0.33*	-0.33*	+0.11	+0.76**	-0.28		
	Wall protein (7)	-0.43 **	+0.36*	-0.36*	+0.43**	-0.21	+0.65**	-0.31	
	R.F.V.	+0.32 *	-0.59**	+0.59**	-0.41**	+0.10	-0.21	-0.11	-0.20

* Correlation significantly different at the 5 percent level. ** Correlation significantly different at the 1 percent level.

Table 23. Correlation coefficient between the ratio of leaf to stem and the feeding value variables as well as among the cell wall constituents in forage of each studied legume in Exp. I.

Species	Variables	Leaf-Stem ratio	(1)	(2)	(3)	(4)	(5)	(6)	(7)
Alfalfa	NDF (1)	+ 0.41							
	Cellular content (2)	- 0.41	- 1.00**						
	ADF (3)	+ 0.38	+ 0.86**	-0.86**					
	Hemicelluloses (4)	- 0.20	+ 0.40	-0.40	+0.07				
	p-Lignin (5)	+ 0.27	+ 0.48*	-0.48*	+0.60**	-0.27			
	Cellulose (6)	- 0.20	+ 0.38	-0.38	+0.16	+0.76**	-0.41		
	Wall protein (7)	+ 0.03	- 0.21	+0.21	-0.12	-0.44	+0.18	-0.49*	
	RFV (8)	- 0.14	- 0.47*	+0.47*	-0.45*	-0.27	+0.04	-0.28	+0.06
Trefoil	NDF (1)	- 0.93**							
	Cellular content (2)	+ 0.93**							
	ADF (3)	- 0.71**	+ 0.71**	-0.71**					
	Hemicelluloses (4)	- 0.60**	+ 0.57**	-0.57**	+0.07				
	p-Lignin (5)	- 0.46*	+ 0.48*	-0.48*	+0.64**	-0.01			
	Cellulose (6)	- 0.64**	+ 0.56*	-0.56*	+0.16	+0.84**	-0.07		
	Wall protein (7)	- 0.25	+ 0.14	-0.14	+0.54*	-0.40	+0.29	-0.18	
	RFV (8)	+ 0.43	- 0.55*	+0.55*	+0.29	-0.16	-0.26	-0.05	+0.12

* Correlation significantly different at the 5 percent level ** Correlation significantly different at the 1 percent level.

tion increased in trefoil and decreased in alfalfa (Table 24). Conversely, the cellular content of trefoil decreased and of alfalfa increased during the corresponding periods (Table 24). Two hypothesis could be proposed to explain the above results: a) the remobilization of nutrients through the inflorescences, in the case of trefoil, and b) the remobilization of nutrient through the leaf fraction.

Alfalfa with 14, 16, 18, 20 and 28 nodes and trefoil ranging from 10 to 24 nodes showed similar trends of changes of NDF and cellular content with age (Table 25 and 26).

Comparison between both legumes

Early bud alfalfa with 16, 18 and 20 nodes had a greater NDF level and a lower cell solubles content than the corresponding stage of trefoil (Table 25, 26). At full bloom, trefoil had both a greater NDF level and lower cell solubles than alfalfa (Table 25 and 26).

ADF

The ADF fraction was not affected by the species, the maturity and the morphological development (Table 20, 27).

Hemicelluloses

The level of hemicelluloses was affected by the stage of morphological development. Both species with high node numbers (24, 26 and 28 nodes) had lower hemicelluloses than in less developed plants (24 nodes) (Table 28). Hemicelluloses were negatively correlated with the leaf-stem ratio of both species ($r = -0.47$, $p < 0.01$, Table 22), mainly influenced by the trend in trefoil ($r = -0.40$, $p < 0.01$, Table 23).

Table 24. Changes in the mean content (% of dry matter) of the various variables of feeding value and the RFV of forage of alfalfa and trefoil with maturity (Exp.I).

Species	Variables	Maturity stages	
		Early Bud	Full Bloom
Alfalfa	NDF	42.4 *	40.8 *
	Cellular content	57.6 *	59.2 *
	ADF	30.7	29.7
	Hemicelluloses	12.0	11.5
	p-Lignin	7.9	8.1
	Cellulose	18.5	17.5
	Wall crude protein	2.28	2.51
	R.F.V.	136.8 *	145.5 *
Trefoil	NDF	41.9 *	46.1 *
	Cellular content	58.1 *	53.9 *
	ADF	29.5 *	33.0 *
	Hemicelluloses	11.6	12.5
	p-Lignin	8.1 *	9.1 *
	Cellulose	16.9	18.1
	Wall crude protein	2.96 *	3.47 *
	R.F.V.	138.2 *	133.1 *

Comparisons between maturity stages are done by the t-test and the means that showed difference at the 5 percent level are followed by *

Table 25. Changes in the level of NDF (% of dry matter) among the various morphological groups of both alfalfa and birdsfoot trefoil, at early bud and full bloom (Exp.I).

Species	Develop. Stages / Maturity stages	10	12	14	16	18	20	22	24	26	28
Alfalfa	Early bud	38.9 e	40.6 de	43.5 abc	45.0 a	43.9 ab	44.0 ab	41.7 dc	42.3 bcd	41.1 d	43.2abcd
	Full bloom	38.2 c	38.9 bc	39.2 abc	40.8 abc	41.9 ab	41.2 abc	42.5 a	42.0 ab	42.6 a	40.4 b
	Average	38.6 bc	39.7 c	41.4 ab	42.9 a	42.9 a	42.6 a	42.1 a	42.2 a	41.9 a	41.8 a
Trefoil	Early bud	41.4 a	42.5 a	42.6 a	41.4 a	41.8 a	40.9 a	42.4 a	41.1 a	41.6 a	43.2 a
	Full bloom	45.8 a	47.1 ab	46.1 ab	47.4 b	47.9 b	47.1 ab	46.1 ab	45.0 ab	44.7 ab	43.8 a
	Average	43.6 a	44.8 a	44.4 a	44.4 a	44.9 a	44.0 a	44.3 a	43.1 a	43.2 a	43.5 a
Average		41.1 A	42.3 A	42.8 A	43.6 A	43.9 A	43.3 A	43.2 A	42.6 A	42.5 A	42.6 A

Any two morphological groups within the same maturity stage and the average with the same letter are not significantly different at the 5 percent level by the Duncan's Multiple Range test.

Table 26. Changes in the level of cellular content among the various morphological groups of both alfalfa and birdsfoot trefoil, at early bud and at full bloom.(Exp. I).

Species	Develop. Stages / Maturity stages	10	12	14	16	18	20	22	24	26	28
Alfalfa	Early bud	61.1 a	59.4 ab	56.5 cde	55.0 e	56.1 de	56.0 de	58.3 bc	57.7 bcd	58.9 b	56.8abcd
	Full bloom	61.8 a	61.1 ab	60.8 abc	59.2 abc	58.1 bc	58.8 abc	57.5 c	58.0 bc	57.4 c	59.6 abc
	Average	61.4 a	60.2 ab	58.6 bc	57.1 c	57.1 c	57.4 c	57.9 bc	57.8 bc	58.1 bc	58.2 bc
Trefoil	Early bud	58.6 a	57.5 a	57.4 a	58.6 a	58.2 a	59.1 a	57.6 a	58.9 a	58.4 a	56.8 a
	Full bloom	54.2 ab	52.9 ab	53.9 ab	52.6 b	52.1 b	52.9 ab	53.9 ab	55.0 ab	55.3 ab	56.2 a
	Average	56.4 a	55.2 a	55.6 a	55.5 a	55.4 a	56.2 a	55.7 a	56.9 a	56.8 a	56.5 a
Average		58.9 A	57.7 A	57.1 A	56.3 A	56.2 A	56.8 A	56.8 A	57.4 A	57.5 A	57.3 A

Any two morphological groups within the same maturity stage and the average with the same letter are not significantly different at the 5 percent level by the Duncan's Multiple Range test.

Table 27. Changes in the level of ADF among the various morphological groups of both alfalfa and birdsfoot trefoil, at early bud and at full bloom (Exp. I).

Species	Develop. Stages / Maturity stages	10	12	14	16	18	20	22	24	26	28
Alfalfa	Early bud	27.2 b	29.9 a	31.2 a	32.8 a	31.8 a	30.3 a	30.0 a	31.6 a	31.1 a	----
	Full bloom	27.2 e	28.0 de	28.4 de	28.9 cde	31.1 abc	30.1abcd	29.8 bcd	31.4 ab	32.5 a	29.0 cd
	Average	27.2 c	29.0 bc	29.8 ab	30.8 ab	31.5 a	30.2 ab	29.9 ab	31.5 a	31.8 a	29.0 ab
Trefoil	Early bud	29.1 a	30.2 a	30.6 a	30.1 a	30.2 a	27.3 a	28.7 a	29.5 a	31.1 a	----
	Full bloom	32.9 a	32.5 a	31.7 a	34.0 a	34.2 a	31.4 a	31.7 a	32.6 a	33.3 a	35.3 a
	Average	31.0 a	31.3 a	31.1 a	32.1 a	32.2 a	29.4 a	30.2 a	31.1 a	32.2 a	35.3 a
Average		29.1 B	30.1 AB	30.4 AB	31.4 A	31.8 A	29.8 AB	30.0 AB	31.3 AB	32.0 A	32.1 A

Any two morphological groups within the same maturity stage and the average with the same letter are not significantly different at the 5 percent level by the Duncan's Multiple Range test.

Table 28. Changes in the level of hemicelluloses among the various morphological groups of both alfalfa and birdsfoot trefoil, at early bud and at full bloom (Exp. I).

Species	Develop. Stages / Maturity stages	10	12	14	16	18	20	22	24	26	28
Alfalfa	Early bud	12.1 bcd	11.7 bcd	13.4 ab	12.8 abc	14.2 a	12.4 bcd	11.2 cde	11.0 de	9.8 e	----
	Full bloom	11.9abcd	11.7abcd	11.1 bcd	12.2 abc	12.7 ab	10.5 cd	12.9 a	10.9 cd	10.3 d	10.6 cd
	Average	12.0 abc	11.7 bc	12.3 abc	12.5 ab	13.5 a	11.5 bcd	12.1 abc	10.9 cd	10.1 d	10.6 bcd
Trefoil	Early bud	12.0 ab	12.9 a	11.6 ab	12.3 a	12.4 a	11.6 ab	12.0 ab	10.5 bc	9.5 c	----
	Full bloom	12.3 ab	14.6 a	13.4 ab	12.5 ab	14.1 a	13.3 ab	13.4 ab	11.4 bc	9.7 c	10.1 c
	Average	12.2 ab	13.8 a	12.5 ab	12.4 ab	13.2 a	12.5 ab	12.7 a	11.0 bc	9.6 c	10.1 bc
Average		12.1AB	12.7AB	12.4AB	12.4AB	13.3A	12.0BC	12.4AB	10.9C	9.8D	10.3CD

Any two morphological groups within the same maturity stage and the average with the same letter are not significantly different at the 5 percent level by the Duncan's Multiple Range test.

Permanganate-lignin (p-lignin)

The level of p-lignin increased from early bud to full bloom, in most groups of plant development of trefoil (Table 29). In contrast, the p-lignin level generally remained unchanged in alfalfa with maturity. These results followed the changes in morphology, especially of the leaf to stem (Chapter I, Exp.I), that would affect the proportions of the various types of cell wall present.

At early bud, trefoil had a higher level of p-lignin in the wall of its plants with 12 and 18 nodes than in the wall of corresponding alfalfa (Table 29) but alfalfa with 20 and 26 nodes had a higher level of p-lignin than trefoil. At full bloom, alfalfa of nodal group 10, 12 and 16 contained less p-lignin than the corresponding group of birdsfoot trefoil (Table 29).

Cellulose

The cellulose level of the legumes was affected by the stage of development. Plants with the highest number of nodes had the lowest cellulose level (Table 31). Cellulose was negatively correlated to the leaf-stem ratio ($r = -0.34$, $p < 0.05$, Table 22). Trefoil cellulose was the most affected by the proportion of plant parts ($r = -0.64$, $p < 0.01$, Table 23). These results indicated that the level of cellulose was generally closely influenced by the cellulose contained in the stem fraction. The leaves diluted the cellulose fraction of the total.

Cell wall protein

The content of wall crude protein was influenced by the species. At every maturity stage, trefoil had a higher wall crude protein than al-

Table 29. Changes in the level of p-lignin among the various morphological groups of both alfalfa and birdsfoot trefoil at early bud and at full bloom (Exp. I).

Species	Develop. Stages / Maturity stages	10	12	14	16	18	20	22	24	26	28
Alfalfa	Early bud	5.9 b	6.2 b	8.3 a	8.6 a	8.1 a	8.2 a	8.2 a	8.1 a	9.1 a	----
	Full bloom	8.0 bc	6.9 c	7.8 bc	7.4 bc	8.1 bc	8.6 ab	8.2 b	9.4 a	8.6 ab	8.5 ab
	Average	7.0 b	6.5 b	8.1 a	8.0 a	8.1 a	8.4 a	8.2 a	8.8 a	8.9 a	8.5 a
Trefoil	Early bud	7.3 c	7.9 abc	8.8 ab	8.0 abc	9.1 a	7.4 c	7.6 bc	9.1 a	8.4 abc	----
	Full bloom	10.4 a	9.4 ab	7.6 b	10.1 a	8.7 ab	9.1 ab	8.4 ab	8.9 ab	8.5 ab	9.8 a
	Average	8.8 a	8.7 a	8.2 a	9.1 a	8.9 a	8.2 a	8.0 a	9.0 a	8.4 a	9.8 a
Average		7.9 AB	7.6B	8.1AB	8.5AB	8.5AB	8.3AB	8.1AB	8.9A	8.6AB	9.1A

Any two morphological groups within the same maturity stage and the average with the same letter are not significantly different at the 5 percent level by the Duncan's Multiple Range test.

Table 30. Changes in the level of cellulose among the various morphological groups of both alfalfa and birdsfoot trefoil at early bud and at full bloom (Exp. I).

Species	Maturity stages	10	12	14	16	18	20	22	24	26	28
Alfalfa	Early bud	18.5 abc	20.7 ab	18.3 abc	20.6 ab	21.2 a	19.2 abc	17.4 bc	16.3 cd	14.1 d	----
	Full bloom	16.7 bc	17.9 abc	17.9 abc	17.3 abc	19.4 a	18.4 abc	18.6 ab	15.9 c	17.0 abc	15.6 c
	Average	17.6 bcd	19.3 ab	18.1 abc	19.0 ab	20.3 a	18.8 ab	18.0 abc	16.1 cd	15.6 d	15.6 d
Trefoil	Early bud	19.3 a	18.3 ab	17.2 abc	15.5 bc	18.2 ab	17.1 abc	17.2 abc	14.2 c	14.7 c	----
	Full bloom	18.1 abc	19.0 ab	19.1 ab	18.0 abc	20.4 a	18.1 abc	19.9 a	16.4 bc	15.4 c	16.2 c
	Average	18.7 ab	18.7 ab	18.2 ab	16.7 bc	19.3 a	17.6 ab	18.6 ab	15.3 c	15.1 c	16.2 bc
Average		18.1ABC	19.0 AB	18.1 ABC	17.8ABCD	19.8 A	18.2 ABC	18.3 ABC	15.7 CD	15.3 D	15.9 CD

Any two morphological groups within the same maturity stage and the average with the same letter are not significantly different at the 5 percent level by the Duncan's Multiple Range test.

Table 31. Changes in the level of wall crude protein (%) among the various morphological groups of both alfalfa and birdsfoot trefoil, at early bud and at full bloom (Exp. I).

Species	Develop. Stages / Maturity stages	10	12	14	16	18	20	22	24	26	28
Alfalfa	Early bud	2.19 bc	2.03 c	2.18 bc	2.03 c	2.06 c	2.11 c	2.23 bc	2.31 bc	2.53 ab	3.10 a
	Full bloom	2.23 b	2.50 ab	2.66 ab	2.56 ab	2.49 ab	2.38 b	2.29 b	2.34 b	2.68 ab	3.02 a
	Average	2.21 b	2.27 b	2.42 b	2.30 b	2.27 b	2.24 b	2.26 b	2.32 b	2.61 b	3.06 a
Trefoil	Early bud	3.26 a	2.78 a	2.90 a	2.89 a	3.06 a	3.16 a	2.69 a	3.11 a	2.85 a	2.75 a
	Full bloom	3.01 de	3.06 cde	2.81 e	3.10 cde	2.88 e	3.43 bcd	3.67 b	3.49 bc	4.25 ab	4.97 a
	Average	3.14 a	2.92 a	2.86 a	3.00 a	2.97 a	3.29 a	3.18 a	3.30 a	3.55 a	3.86 a
Average		2.67 B	2.59 B	2.64 B	2.65 B	2.62 B	2.77 B	2.72 B	2.81 AB	3.08 AB	3.46 A

Any two morphological groups within the same maturity stage and the average with the same letter are not significantly different at the 5 percent level by the Duncan's Multiple Range test.

Table 32. Changes of the forage feed relative value among the various morphological groups of both alfalfa and birdsfoot trefoil, at early bud and at full bloom (Exp. I).

Species	Develop. Stages / Maturity stages	10	12	14	16	18	20	22	24	26	28
Alfalfa	Early bud	145.1 a	139.9 b	134.1 cd	129.5 d	134.1 cd	134.8 bc	138.6 bc	135.6 bc	139.5 b	----
	Full bloom	145.6 b	144.1 bc	143.2 bc	141.4bcd	136.7 cd	155.3 a	156.1 a	156.8 a	133.7 d	141.9 c
	Average	145.3 a	142.0 a	138.7 a	135.5 a	135.4 a	145.1 a	147.3 a	146.2 a	136.6 a	141.9 a
Trefoil	Early bud	140.2 a	137.3 a	139.5 a	139.6 a	142.7 a	139.2 a	139.6 a	136.3 a	139.5 a	----
	Full bloom	136.7 a	129.1 a	131.9 a	121.4 a	134.5 a	136.7 a	138.8 a	129.2 a	127.0 a	145.1 a
	Average	138.5 a	133.2 a	135.7 a	130.5 a	138.6 a	137.9 a	139.2 a	132.8 a	133.2 a	145.1 a
Average		141.9 AB	137.6 AB	137.2 AB	133.0 B	137.0 AB	141.5 Ab	143.3 A	139.5 AB	134.9 AB	137.9 AB

Any two morphological groups within the same maturity stage and the average with the same letter are not significantly different at the 5 percent level by the Duncan's Multiple Range test.

Table 33. Analysis of variance of the factors: species (S), maturity stages (M) and plant layers (P) and their interactions on the content of various feeding variables in leaf of plants with 18 nodes development (Exp. II).

Variables	Main Effects and Interactions						
	S	M	P	S x M	S x P	M x P	S x M x P
NDF	**	N.S.	*	N.S.	N.S.	N.S.	N.S.
Cellular contents	**	N.S.	*	N.S.	N.S.	N.S.	N.S.
ADF	**	N.S.	**	N.S.	N.S.	N.S.	N.S.
Hemicelluloses	N.S.	**	N.S.	N.S.	N.S.	N.S.	N.S.
p-Lignin	**	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
Celluloses	*	N.S.	*	N.S.	N.S.	N.S.	N.S.
Wall crude protein	**	N.S.	N.S.	N.S.	*	*	N.S.
R.F.V.	**	N.S.	**	N.S.	N.S.	N.S.	N.S.

The significance of factors was evaluated at 1 percent level and followed by ** and by * at 5 percent level and N.S. followed those which are not significant.

Table 34. The RFV and the mean levels (% of dry matter) of feeding variables in leaf and stem of alfalfa and birdsfoot trefoil (Exp. II).

Plant parts	Variables	Species	
		Alfalfa	Trefoil
Leaf	NDF	16.4 **	17.8 **
	Cellular contents	83.6 **	82.2 **
	ADF	11.1 **	12.7 **
	Hemicelluloses	5.0	5.3
	p-Lignin	2.1 **	4.2 **
	Celluloses	6.9 *	6.1 *
	Wall crude protein	6.37 **	10.30 **
	R.F.V.	131.1 **	135.1 **
Stem	NDF	53.1 *	54.8 *
	Cellular contents	46.9 *	45.2 *
	ADF	36.9 **	40.0 **
	Hemicelluloses	16.5	15.4
	p-Lignin	8.7	9.2
	Celluloses	24.1 *	24.9 *
	Wall crude protein	1.96 **	2.34 **
	R.F.V.	88.3	90.1

Comparisons between both species are done by the t-test and the means that showed difference at 5 percent level are followed by * and by ** at the 1 percent level.

falfa (Table 21, 24). The levels of wall crude protein and lignin were correlated which would indicate the existence of a link between both cell wall components.

The comparison of the wall crude protein between the various development groups of both species in the early bud stage showed higher level of nitrogen in almost all groups of trefoil than alfalfa (Table 31). In full bloom plants, trefoil with 20 or more nodes had a higher wall protein fraction than alfalfa. The nitrogen in the cell wall seems to be highly influenced by the physiological age of the plant: the longer a plant delays in reaching a specific stage, the greater is its wall protein.

Relative Feeding Value

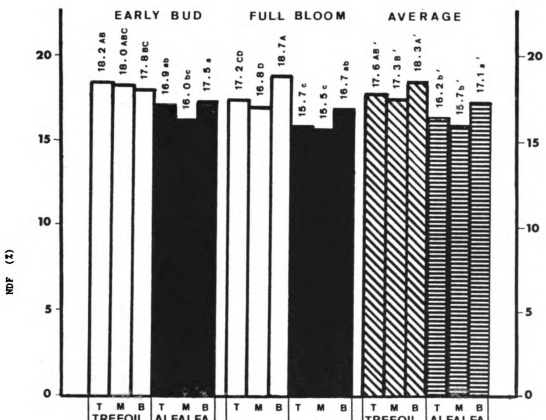
The RFV of both species changed slightly with the stage of maturity but it was not affected by the stage of development as well as by the species (Table 24).

EXPERIMENT II

NDF

Birdsfoot trefoil leaves had higher NDF (17.8%) than alfalfa (16.4%) Table 34. Trefoil leaves had a higher NDF content than alfalfa (Fig. 22) at most plant layers of every growth stage except that the basal leaves of both early bud trefoil and alfalfa had a comparable NDF level.

A possible association could exist between the NDF and the thickness of the epidermal layer and the thickness of the mesophyll cell wall. Lees et al (1982) found a thicker epidermal layer in a bloat-safe



VERTICAL LAYERS T: top (1-6th nodes) M: middle (7-12th nodes) B: bottom (13-18th nodes)

Fig. 22 Vertical distribution of NDF in leaf of both alfalfa and birdsfoot trefoil at two maturity stages (Exp. II).

Comparisons between layers and maturity stages are shown by capital letter within trefoil and by small letter within alfalfa and the means with the same letter are not different at the 5 percent level by the Duncan's Multiple Range test.

Comparisons between layers of the bulk data within each species are shown by capital letter with ' for trefoil and by small letters with ' for alfalfa.

birdsfoot trefoil than in a bloat-causing alfalfa. In contrast, Howarth et al (1978) found that the cell wall of the mesophyll cells had a greater mechanical resistance which meant a denser wall that could possibly have a positive influence on the level of NDF.

The content of NDF in leaf of the two upper layers was stable at approximately 16.9% (Table 35) and rose to 17.7% in the basal leaves.

The stem NDF was affected by: a) the species, b) the stem position, c) the species x plant layers interaction, and d) the species x maturity stage interaction (Table 36).

The NDF in trefoil stems was greater (at $p < 0.05$) than in alfalfa (Table 34). Full bloom trefoil stem had 55.3% of its dry matter in structural components while the alfalfa stem had 51.3% of its dry matter in NDF (Table 37). The difference of the NDF level between both species was mainly through the amount of sclerenchyma for which trefoil showed a positive correlation with the NDF level ($r = +0.42$, $p < 0.05$).

The NDF level increased from the plant top (37.3%) to bottom (66.5%) as an average for both legume (Table 35). A similar trend was noticed within each species and at each maturity stage with the exception that the basal stem of full bloom trefoil and the median portion had a similar NDF.

The level of NDF in the stem of both legumes varied depending upon the stem position on the plant. Thus, the upper and the median stem of trefoil had a mean NDF content higher than that of alfalfa (Fig. 23). The high level of NDF in the stem of trefoil and alfalfa at these two plant layers was attributable to the high amount of the

Table 35. Vertical changes of the mean level of various feeding variables in leaf and in stem of both alfalfa and birdsfoot trefoil (Exp. II).

Plant parts	Variables	Vertical Layers		
		TOP (1-6 th)	MIDDLE (7-12 th)	BOTTOM (13-18 th)
Leaf	NDF	16.9 b	16.5 b	17.7 a
	Cellular contents	83.1 a	83.5 a	82.3 b
	ADF	11.1 b	11.2 b	13.3 a
	Hemicelluloses	5.6 a	5.5 a	4.2 b
	p-Lignin	3.0 a	3.2 a	2.9 a
	Celluloses	5.8 c	6.3 b	7.2 a
	Wall crude protein	9.01 a	8.24 b	7.74 b
	R.F.V.	132.2 b	131.3 b	135.5 a
Stem	NDF	37.3 c	58.0 b	66.5 a
	Cellular contents	62.7 a	42.0 b	33.5 c
	ADF	25.7 c	40.7 b	48.9 a
	Hemicelluloses	12.0 b	17.5 a	18.1 a
	p-Lignin	5.7 c	9.1 b	11.9 a
	Celluloses	17.3 c	26.2 b	29.9 a
	Wall crude protein	2.65 a	1.87 b	1.90 b
	R.F.V.	143.3 a	83.0 b	41.4 c

Comparisons between layers on the bulk data of both species within each variable of each plant part are shown by small letters and the means with the same letter are not significantly different at the 5 percent level by the Duncan's test.

Table 36. Analysis of variance of the factors: species (S), maturity stages (M) and plant layers (P) and their interactions on the level of various feeding variables in stem of plant with 18 nodes development (Exp. II).

Variables	Main Effects and Interactions						
	S	M	P	S x M	S x P	M x P	S x M x P
NDF	*	N.S.	**	**	**	N.S.	N.S.
Cellular contents	*	N.S.	**	**	**	N.S.	N.S.
ADF	**	N.S.	**	N.S.	**	N.S.	N.S.
Hemicelluloses	N.S.	N.S.	**	N.S.	**	N.S.	N.S.
p-Lignin	N.S.	N.S.	**	N.S.	**	N.S.	N.S.
Celluloses	*	N.S.	**	*	**	**	**
Wall crude protein	**	N.S.	**	**	**	**	**
R.F.V.	N.S.	N.S.	**	N.S.	**	N.S.	N.S.

The significance of factors was evaluated at 1 percent level and followed by ** and by * at 5 percent level and N.S. followed those which are not significant.

Table 37. The RFV and the mean level of various feeding variables in leaf and in stem of alfalfa and birdsfoot trefoil at the two studied maturity stages (Exp. II).

Species	Plant parts	Variables	Maturity Stages	
			Early Bud	Full Bloom
Alfalfa	Leaf	NDF	16.8	16.0
		Cellular content	83.2	84.0
		ADF	11.4	10.8
		Hemicelluloses	5.1	4.9
		p-Lignin	1.9	2.2
		Cellulose	7.1	6.7
		Wall crude protein	6.57	6.16
		R.F.V.	132.3 *	129.9 *
	Stem	NDF	54.9 **	51.3 **
		Cellular content	45.1 **	48.7 **
		ADF	38.2 *	35.7 *
		Hemicelluloses	16.9	16.1
		p-Lignin	9.0	8.4
		Cellulose	24.3	24.0
		Wall crude protein	2.05 *	1.88 *
		R.F.V.	84.3 **	92.5 **
Trefoil	Leaf	NDF	18.0	17.6
		Cellular content	82.0	82.4
		ADF	12.6	12.8
		Hemicelluloses	5.8 **	4.7 **
		p-Lignin	4.4	3.9
		Cellulose	5.9	6.3
		Wall crude protein	10.46	10.15
		R.F.V.	135.6	134.6
	Stem	NDF	54.3	55.3
		Cellular content	45.7	44.7
		ADF	40.1	40.0
		Hemicelluloses	14.9	15.9
		p-Lignin	9.2	9.3
		Cellulose	24.2 **	25.6 **
		Wall crude protein	2.14 **	2.54 **
		R.F.V.	92.4 *	87.9 *

Comparisons between maturity stages for each variable and for each plant part of each species are done by the t-test and the means that showed difference at the 5 percent are followed by * and by ** at the 1 percent level.

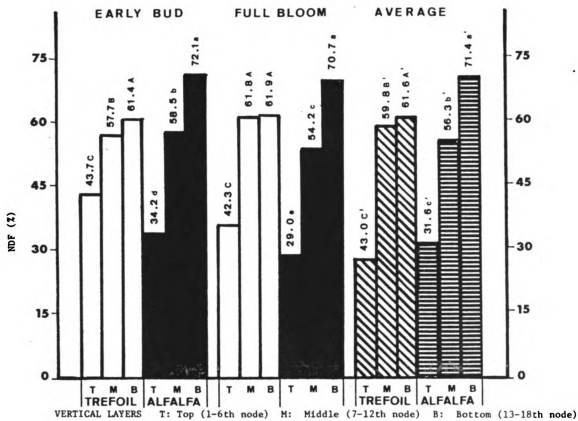


Fig. 23 Vertical distribution of NDF in stem of alfalfa and birdsfoot trefoil at two stages of maturity (Exp. II).

Comparisons between layers and maturity stages are shown by capital letter within trefoil and by small letter within alfalfa and the means with the same letter are not different at the 5 percent level by the Duncan's Multiple Range test.

Comparisons between layers of the bulk data within each species are shown by capital letter with ' for trefoil and by small letters with ' for alfalfa.

primary stems (Chapter I).

The above finding indicated that the trefoil primary stem was the major assimilate sink, although there was some activity of remobilization of nutrients through the development and expansion of secondary-tertiary structures besides the development of reproductive parts at the mature stage.

The NDF of the alfalfa stem declined from early bud (54.9%) to full bloom (51.3%) (Table 37).

The percentage of NDF in the stem was about three times as much as an equal weight of the leaf. The NDF fraction between leaf and stem changed from 1:2 at the top to 1:4 at the middle and 1:4 at the bottom layer.

Alfalfa had a similar pattern of partition of the NDF fraction between leaf and stem. In trefoil, the upper shoot portion showed a ratio of leaf to stem NDF of 1:2 while it stood at 1:3 at the two lower stem segments. For the whole plant, the mean proportion of NDF found in the leaf to that in the stem represented 1:3 on both species.

Cellular contents

The cellular content in the leaf and stem was statiscally influenced by the same factors that affected the NDF fraction (Table 33, 36). The pattern of changes according to the various factors was the inverse of those on the NDF fraction.

ADF

The ADF fraction of the leaf was affected by the species and leaf position. Trefoil averaged 12.7% ADF whereas alfalfa had 11.1%

(Table 34).

The ADF fraction as an average for both species was similar (11.1-11.2%) at the two upper segments and 13.3% at the oldest leaves (Table 35).

Trefoil had a higher ADF level than alfalfa at the early bud stage and in the upper and basal leaves of full bloom (Fig. 26).

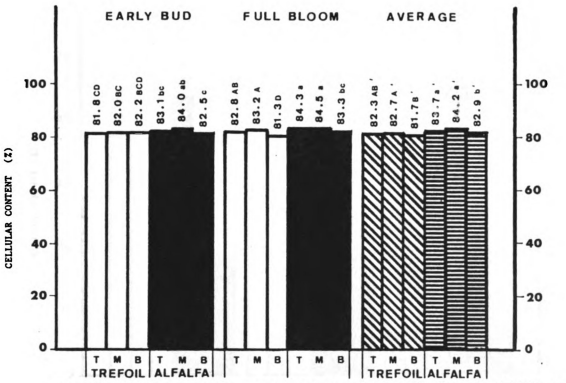
The stem ADF was influenced by the species as well as by the position (Table 34). A species x plant layers interaction had a marked effect on the stem ADF. The birdsfoot trefoil stem had a higher ADF (40.0%) than the stem of alfalfa (36.9%) at each maturity stage (Table 36, 37).

The ADF in stems of both legumes increased from 25.7% at the top to 48.9% at the bottom (Table 35). The trend was similar for each legume and maturity stage (Fig. 27).

The fiber content was greatest in both upper stem segments of trefoil and the basal stem segment of alfalfa (Fig. 27). At the median stem portion, both legumes had comparable ADF fractions.

The concentration of ADF in stems was three times that in leaves. The ratio of stem to leaf ADF increased from 2:1 at the top to 4:1 at the two lower portions corresponding to the high amount of lignified vascular tissues and sclerenchyma of the median and basal segments.

The ratio ADF in stem and leaf was 3:1 for both species. The ratio remained steady at 3:1 at each layer for trefoil. The results suggest that fibrous fraction of alfalfa was mainly affected by lignified xylem while on trefoil the fibrous content was mainly affected by sclerenchyma.

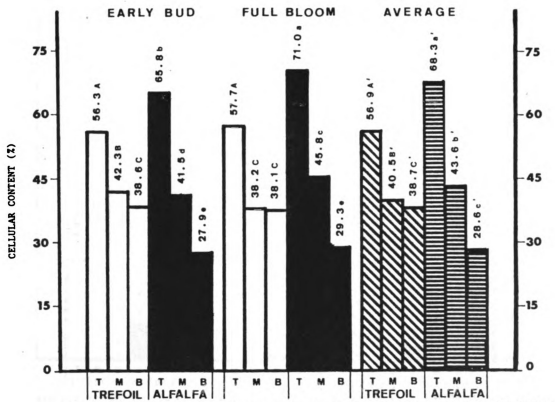


VERTICAL LAYERS T: top (1-6 th nodes) M.: middle (7-12th nodes) B: bottom (13-18th nodes)

Fig. 24 Vertical distribution of cellular content in leaf of both alfa.fa and birdsfoot trefoil at two maturity stages (Exp.II).

Comparisons between layers and maturity stages are shown by capital letter within trefoil and by small letter within alfalfa and the means with the same letter are not different at the 5 percent level by the Duncan's Multiple Range test.

Comparisons between layers of the bulk data within each species are shown by capital letter with ' for trefoil and by small letters with ' for alfalfa.

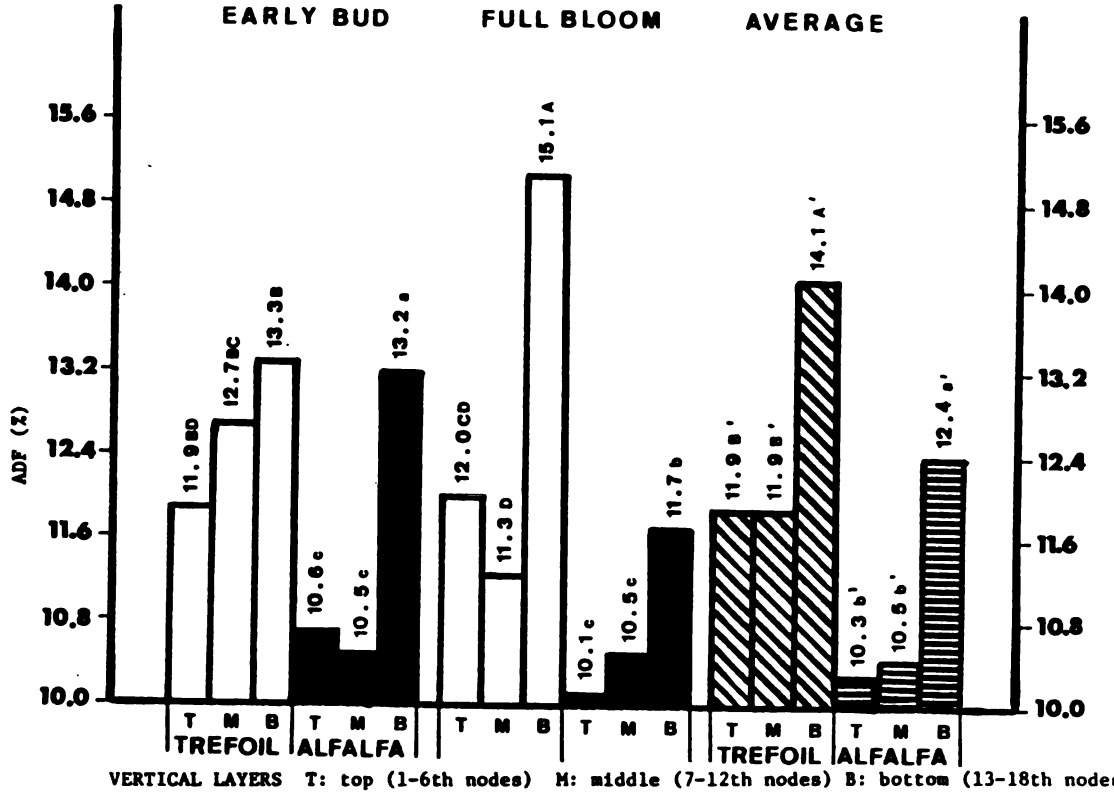


VERTICAL LAYERS T: top (1-6th nodes) M: middle (7-12th nodes) B: bottom (13-18th nodes)

Fig. 25 Vertical distribution of cellular content in stem of both alfalfa and birdsfoot trefoil at two maturity stages (Exp. II).

Comparisons between layers and maturity stages are shown by capital letter within trefoil and by small letter within alfalfa and the means with the same letter are not different at the 5 percent level by the Duncan's Multiple Range test.

Comparisons between layers of the bulk data within each species are shown by capital letter with ' for trefoil and by small letters with ' for alfalfa.



VERTICAL LAYERS T: top (1-6th nodes) M: middle (7-12th nodes) B: bottom (13-18th nodes)

Fig. 26 Vertical distribution of ADF content in leaf of both alfalfa and birdsfoot trefoil at two maturity stages (Exp. II).

Comparisons between layers and maturity stages are shown by capital letter within trefoil and by small letter within alfalfa and the means with the same letter are not different at the 5 percent level by the Duncan's Multiple Range test.

Comparisons between layers of the bulk data within each species are shown by capital letter with ' for trefoil and by small letters with ' for alfalfa.

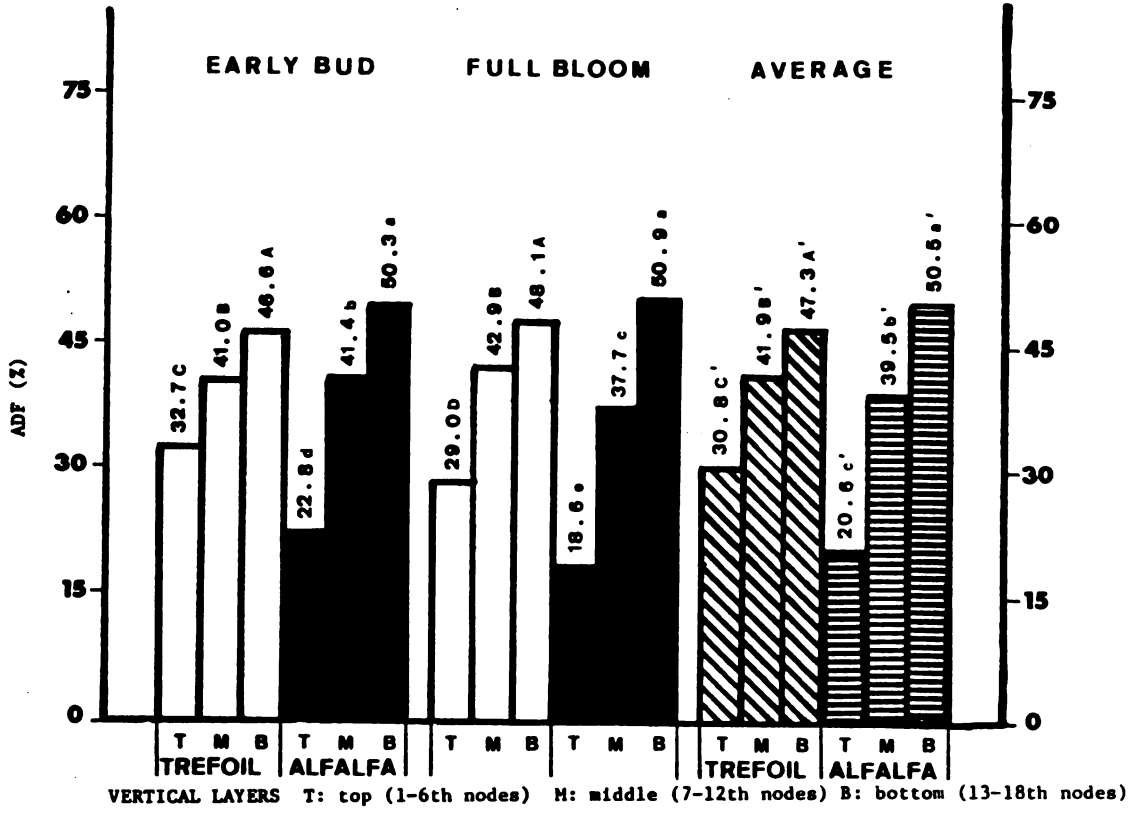


Fig. 27 Vertical distribution of ADF in stem of both alfalfa and birdsfoot trefoil at two maturity stages (Exp. II).

Comparisons between layers and maturity stages are shown by capital letter within trefoil and by small letter within alfalfa and the means with the same letter are not different at the 5 percent level by the Duncan's Multiple Range test.

Comparisons between layers of the bulk data within each species are shown by capital letter with ' for trefoil and by small letters with ' for alfalfa.

Hemicelluloses

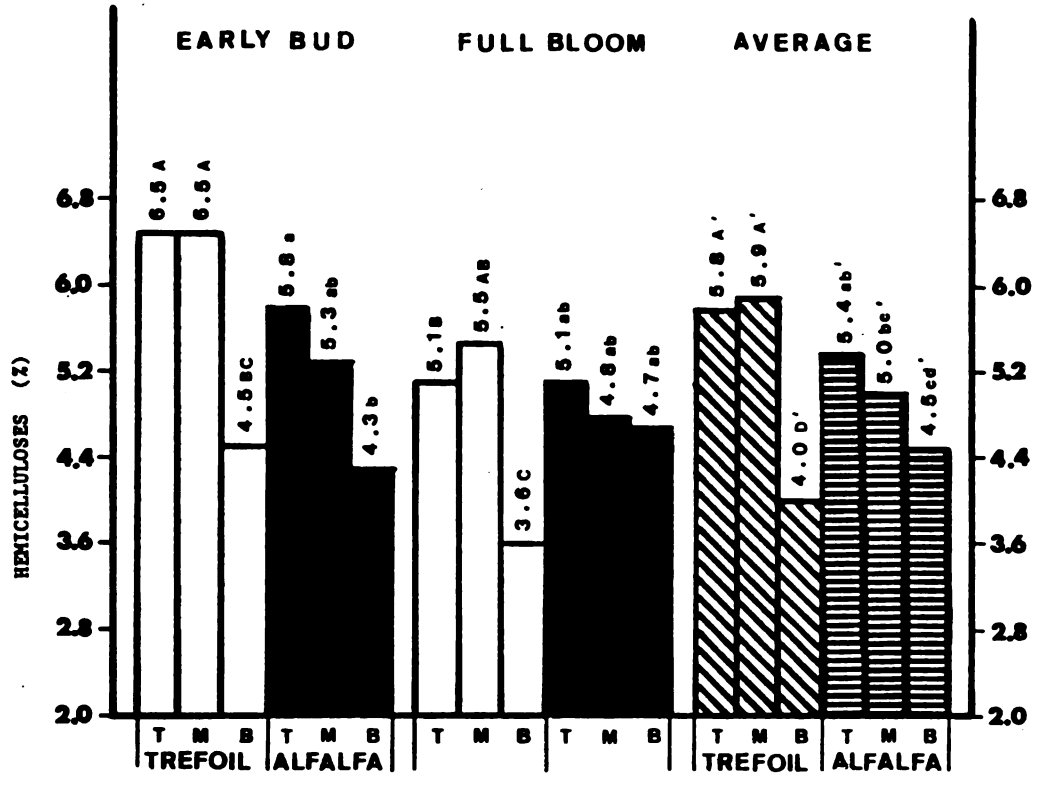
Hemicelluloses in the leaf cell wall were affected primarily by plant maturity. Leaf wall hemicelluloses in trefoil declined from 5.8% at early bud to 4.7% at full bloom (Table 37). Hemicellulose content of alfalfa leaves remained near 5.0% over the two maturity stages. Hemicellulose level was higher in the basal leaves of full bloom alfalfa than trefoil (Fig. 28).

Hemicelluloses in the stem were affected by position on the shoot and by the interaction species x plant layers. The species and the stages of maturity were not determining factors in the content of hemicelluloses in the stem.

Hemicellulose increased from 12.0% at the top to 17.5% at the middle and 18.1% at the bottom layer as an average species (Table 35). Hemicellulose in the upper stem was significantly lower than that of the two lower segments ($p < 0.05$).

Hemicellulose levels in the stem were affected differently by species. In alfalfa, the level of hemicelluloses increased from 10.6% at the top to 21.3% at the bottom layer (Fig. 29). In trefoil, the highest level of hemicellulose was in the median segment (17.7%) while it was 15.0% in the basal and 13.5% in upper stem.

The hemicellulose fraction between stem and leaf for the average of the species was 2:1 at the top, 3:1 at the middle, and 4:1 at the bottom layer. This partition progressed at an equal rate as the from top to bottom as the amount of supporting tissues such as the xylem and the sclerenchyma.



VERTICAL LAYERS T: top (1-6th nodes) M: middle (7-12th nodes) B: bottom (13-18th nodes)

Fig. 28 Vertical distribution of hemicelluloses in leaf of both alfalfa and birdsfoot trefoil at two maturity stages (Exp.II).

Comparisons between layers and maturity stages are shown by capital letter within trefoil and by small letter within alfalfa and the means with the same letter are not different at the 5 percent level by the Duncan's Multiple Range test.

Comparisons between layers of the bulk data within each species are shown by capital letter with ' for trefoil and by small letters with ' for alfalfa.

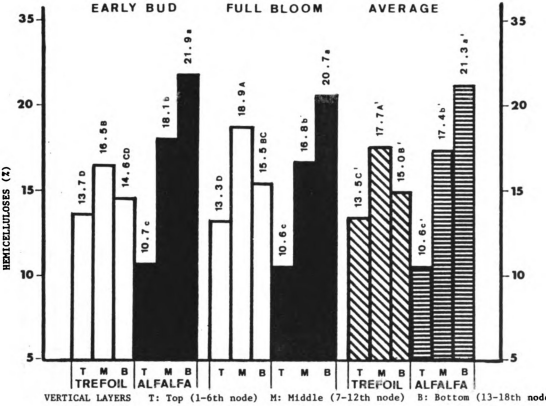


Fig. 29. Vertical distribution of hemicelluloses in stem of alfalfa and birdsfoot trefoil at two maturity stages (Exp. II).

Comparisons between layers and maturity stages are shown by capital letter within trefoil and by small letter within alfalfa and the means with the same letter are not different at the 5 percent level by the Duncan's Multiple Range test.

Comparisons between layers of the bulk data within each species are shown by capital letter with ' for trefoil and by small letters with ' for alfalfa.

The basal stem segment of alfalfa had a higher ratio of hemicelluloses stem:leaf (5:1) than in trefoil.

Permanganate-lignin (p-lignin)

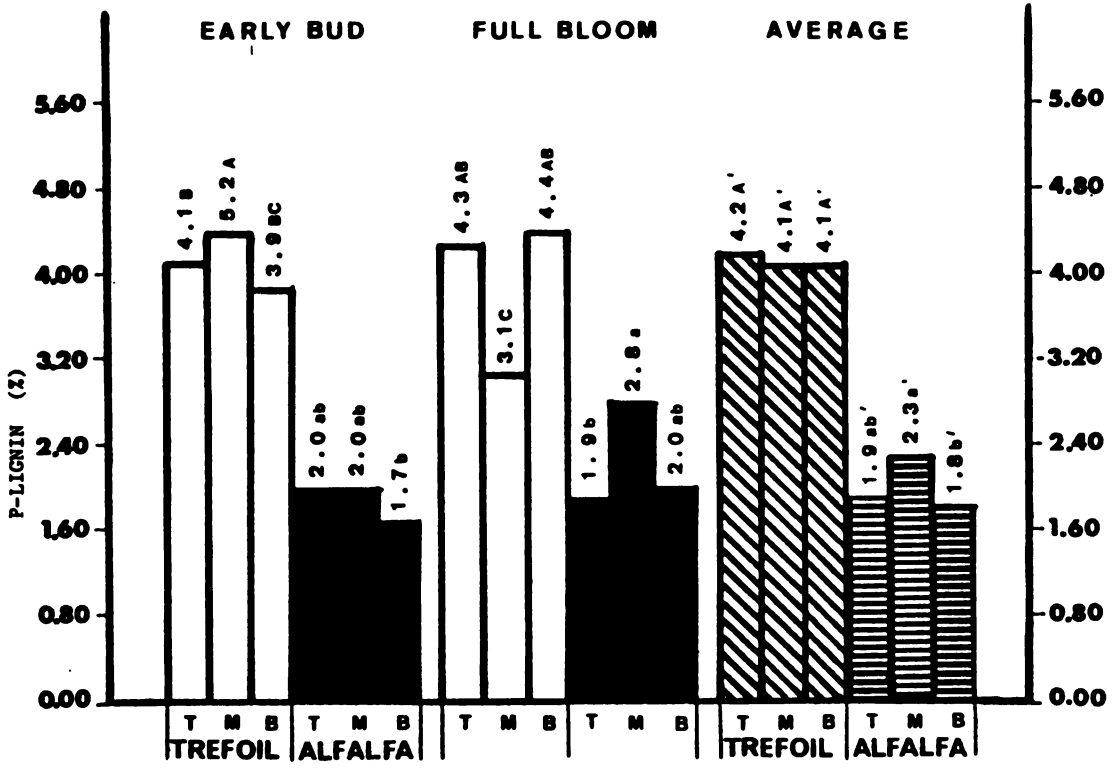
Leaves of trefoil had twice as much p-lignin as in alfalfa at most plant layers and at both stages of maturity (Table 34, 37). Tomlin et al (1965) reported a greater lignin content (Klason lignin) in trefoil than in alfalfa leaves. The high proportion of leaves in the median portion of full bloom alfalfa may have eliminated the gap in the level of p-lignin between legumes. A positive association existed in alfalfa between the leaf contribution and the level of p-lignin ($r = +0.47$, $p < 0.05$).

The position on the shoot was the major factor in the p-lignin of the stem of both species. The combined factors species x plant layers interaction also affected the level of the p-lignin of the stem.

The p-lignin level in the stem increased from the top (5.7%) to bottom (11.9%) (Table 35). The increase of the p-lignin downward was greater in alfalfa than in trefoil (Fig. 31). Because of the coarser and greater stem diameter of alfalfa, the lignification of supporting tissues was likely needed for a greater support of the stem.

The median and basal stem segments of alfalfa had a p-lignin content 200 and 300% higher than that in the upper stem. The p-lignin level in trefoil decreased 21% from top to middle and 43% from top to bottom layer.

Trefoil had a higher p-lignin level than alfalfa in the upper stem (both stages of maturity) and in the median stem (full bloom plants) while, in the basal stem , alfalfa contained about 20% more p-lignin



VERTICAL LAYERS T: top (1-6th nodes) M: middle (7-12th nodes) B: bottom (13-18th nodes)

Fig. 30 Vertical distribution of p-lignin in leaf of both alfalfa and birdsfoot trefoil at two maturity stages (Exp. II).

Comparisons between layers and maturity stages are shown by capital letter within trefoil and by small letter within alfalfa and the means with the same letter are not different at the 5 percent level by the Duncan's Multiple Range test.

Comparisons between layers of the bulk data within each species are shown by capital letter with ' for trefoil and by small letters with ' for alfalfa.

than trefoil (Fig. 31).

From the bulked data, the content of p-lignin in stems almost three times as great as the level in an equal weight of leaves. Those results supported those of Coughman (1960), Bailey et al (1971), and Lopez et al (1966). As the physiological age of the plant advanced, the proportion of p-lignin increased in greater amount in stem than in leaf: 2:1 at the top, 3:1 at the middle, and 4:1 at the bottom layer. The proportion of stem to leaf p-lignin was higher in alfalfa (4:1) than in trefoil (2:1). The vertical distribution on alfalfa was 2:1 at the upper, 4:1 at the median and 7:1 at the basal portion while in trefoil the distribution was 2:1 at the top and middle layer and 3:1 at the bottom.

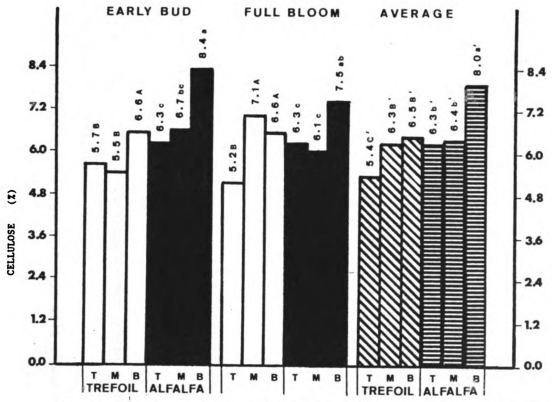
Cellulose

The level of cellulose in leaf cell wall was influenced by the species and the position on the stem. The alfalfa leaf had a slightly higher level of cellulose (6.9%) than trefoil (6.1%) (Table 34). The ratio of leaf width to thickness and the level of cellulose in the leaf cell wall of alfalfa was positively correlated ($r = +0.56$, $p < 0.01$)

Combined data of species at both growth stages showed that the cellulose level in the leaf increased from 5.8% in the upper to 6.3% in the median to 7.2% in the basal segment (Table 35 and Fig. 32).

Cellulose content in the stem was greatly influenced by species and position on the shoot. Interactions between all variables were found to be significant.

The cell wall of the trefoil stem had 24.9% cellulose compared to 24.1% in alfalfa.



VERTICAL LAYERS T: top (1-6th nodes) M: middle (7-12th nodes) B: bottom (13-18th nodes)

Fig . 32 Vertical distribution of cellulose content in leaf of both alfalfa and birdsfoot trefoil (Exp. II).

Comparisons between layers and maturity stages are shown by capital letter within trefoil and by small letter within alfalfa and the means with the same letter are not different at the 5 percent level by the Duncan's Multiple Range test.

Comparisons between layers of the bulk data within each species are shown by capital letter with ' for trefoil and by small letters with ' for alfalfa.

The content of cellulose increased from the top (17.3%) to the bottom layer (29.9%) of the two species (Table 35). In alfalfa, cellulose increased from 13.7% (top) to 32.6% (bottom). With trefoil, the increase of the cellulose occurred between 20.8% at the top and 26.5% at the middle layer (Fig. 33).

Cellulose in the stem was four times that in the leaf of the species. Alfalfa and trefoil had a similar partition of cellulose between stem and leaf.

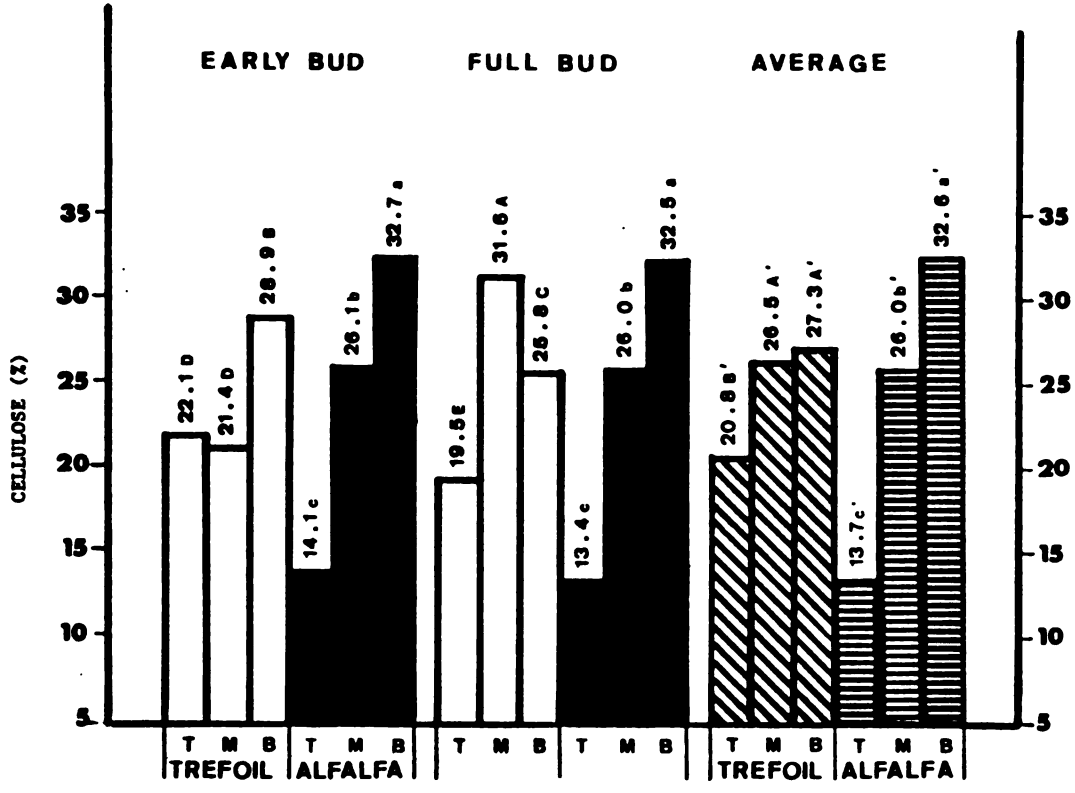
The vertical change in the partition of cellulose in the stem to leaf averaged 2:1 at the top and 4:1 at the middle and bottom layer. Trefoil had a constant ratio of 4:1 cellulose from stem to leaf along the shoot but in alfalfa the ratio was 2:1 at the top and 4:1 at the two lower segments.

Wall crude protein

The leaf cell wall of trefoil contained a mean of 10.30% of crude protein which was higher than the 6.37% in the alfalfa leaf cell wall ($p < 0.01$, Table 34). The high protein level in the cell wall of trefoil may have coincided with the slower growth of that legume as compared to that of alfalfa.

The change of the level of cell wall crude protein in leaves along the shoot was different for each species. Alfalfa leaves declined in crude protein from 9.7% in upper to 7.0% in basal leaves (Fig. 34) but crude protein remained unchanged between layers in trefoil.

The upper leaves of both alfalfa and trefoil declined in wall protein between early bud and full bloom (Fig. 34). No change in the wall crude protein was found in the median level leaves of both alfalfa

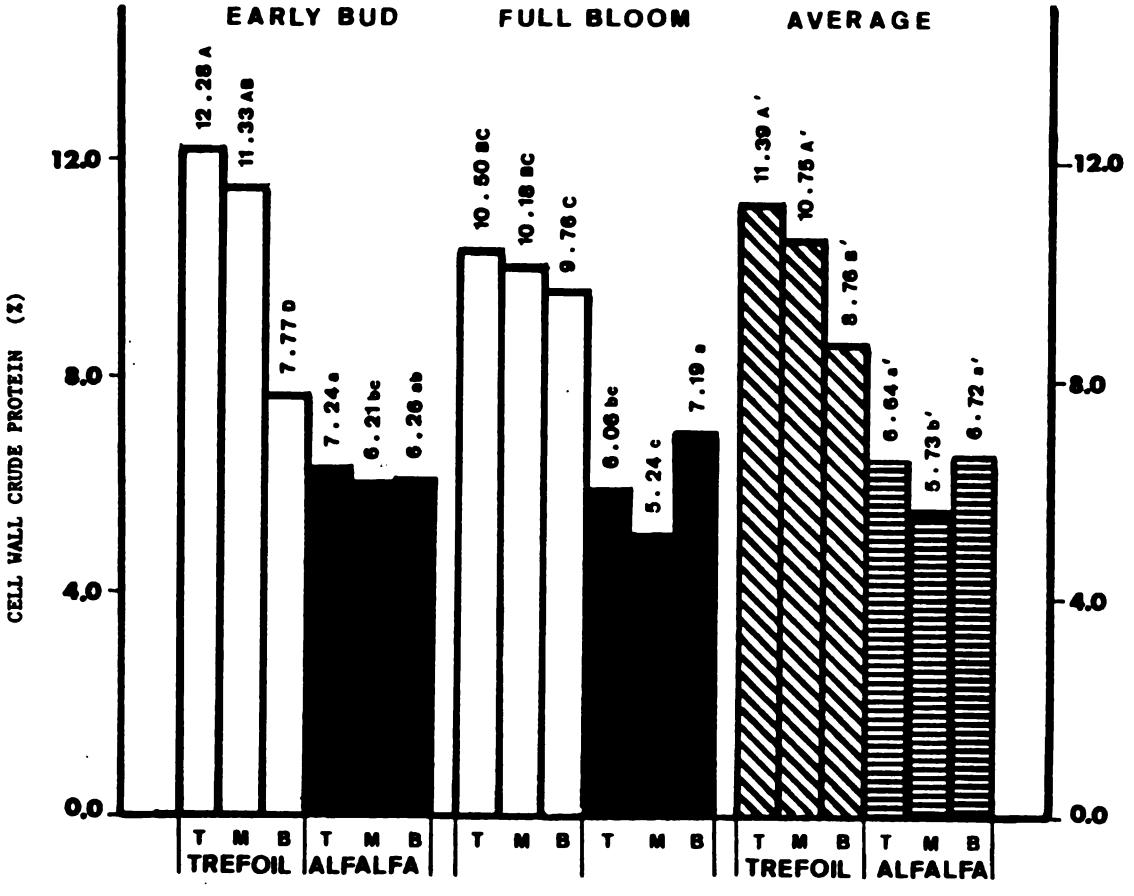


VERTICAL LAYERS T: top (1-6th nodes) M: middle (7-12th nodes) B: bottom (13-18th nodes)

Fig. 33 Vertical distribution of cellulose in stem of both alfalfa and birds-foot at two maturity stages (Exp. II).

Comparisons between layers and maturity stages are shown by capital letter within trefoil and by small letter within alfalfa and the means with the same letter are not different at the 5 percent level by the Duncan's Multiple Range test.

Comparisons between layers of the bulk data within each species are shown by capital letter with ' for trefoil and by small letters with ' for alfalfa.



VERTICAL LAYERS T: top (1-6th nodes) M: middle (7-12th nodes) B: bottom (13-18th nodes)

Fig. 34 Vertical distribution of the cell wall crude protein in leaf of both alfalfa and birdsfoot trefoil at two maturity stages (Exp. II).

Comparisons between layers and maturity stages are shown by capital letter within trefoil and by small letter within alfalfa and the means with the same letter are not different at the 5 percent level by the Duncan's Multiple Range test.

Comparisons between layers of the bulk data within each species are shown by capital letter with ' for trefoil and by small letters with ' for alfalfa.

wall crude protein was found in the median level leaves of both alfalfa and trefoil. An increase ($p < 0.05$) in the level occurred in corresponding leaves of trefoil from early bud (7.77%) to full bloom (9.76%).

The stem of trefoil had a higher level of cell wall protein (2.34%) than alfalfa (1.96%) probably due to its slow growth rate (Table 34). The alfalfa stem contained less crude protein in its cell wall at full bloom than at early bud (2.05%, Table 37) but the trefoil stem increased its cell wall crude protein as the plant matured (from 2.14 to 2.54%). A close and negative association existed between the proportion of stem and the crude protein in stem wall. The latter relationship corresponded, respectively, on alfalfa at $r = -0.76$, $p < 0.01$ and on trefoil at $r = -0.47$, $p < 0.01$, indicating that the increasing stem weight with age might have a diluting effect on the protein. The increase in stem weight may come from the enhancement of branches on alfalfa which diluted the wall protein level of the stem fraction or the enhancement of the wall thickeness on trefoil by accumulation of the structural components.

The crude protein in the cell wall of a young stem segment was higher (2.65%) than in median (1.87%) and basal (1.90%) segments (Table 35).

The level of crude protein in cell walls of leaves was four times higher than in stems for the two species average. The wall crude protein between stem and leaf was 1:3 at the top and 1:4 at the middle and at the bottom layer. The cell walls of alfalfa had a 1:3 ratio of stem to leaf crude protein while trefoil had a 1:4 ratio.

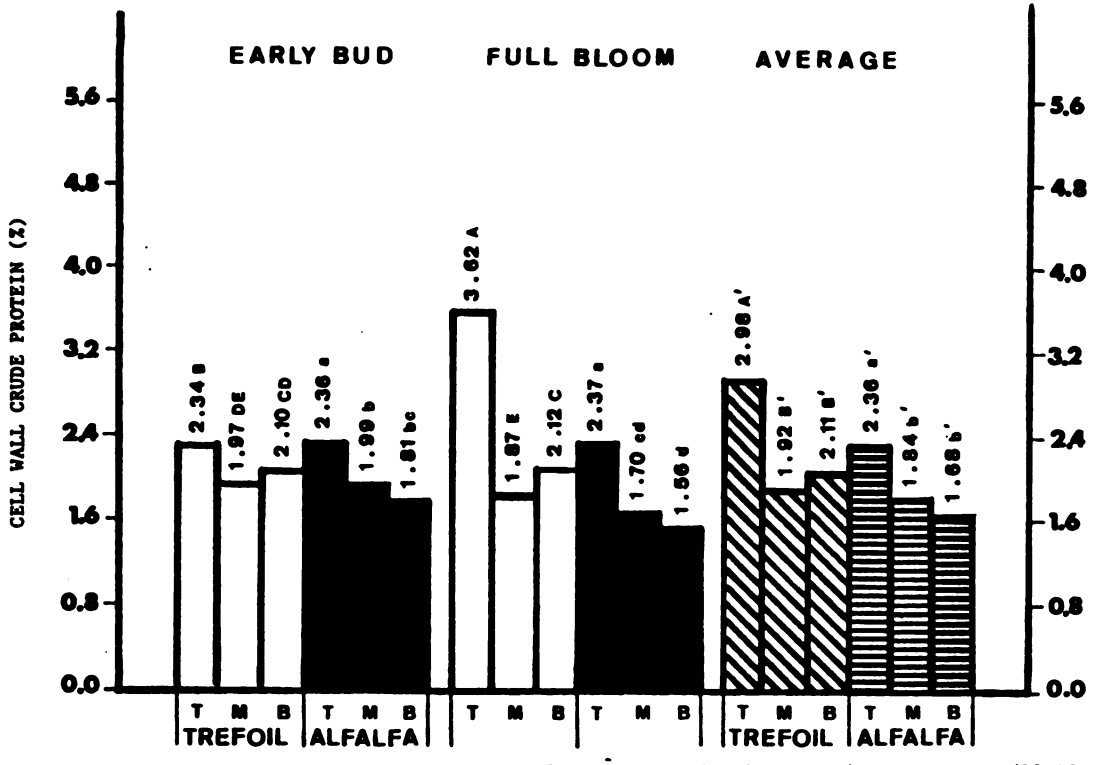
Relative Feeding Value

The RFV of leaves was affected by species and position on the shoot. The maturity stages and the combined factor interactions did not have any effect on the leaf RFV.

The trefoil leaf had a similar RFV (135.1) as alfalfa (131.1, Table 34). The RFV of leaves of the two upper layers was similar but increased for the basal leaves (Table 35). A similar trend was noticed for each species at each maturity stage. except that the leaf RFV of early bud trefoil was stable from plant top to bottom (Fig. 36).

The RFV of both species declined from an average 143.3 at the top to 41.4 at the bottom layer (Table 35). A similar decline in the stem RFV was noticed within each species at each maturity stage (Fig. 37). These results supported those of Terry and Tilley (1964) and Smith (1970).

The upper stem of alfalfa had a greater RFV than of trefoil at corresponding layer at both maturity stages. Conversely, the basal stem of trefoil had a greater RFV than alfalfa, at both growth stages (Fig. 37). At the median segment, the stem RFV of alfalfa was greater than trefoil only at full bloom.

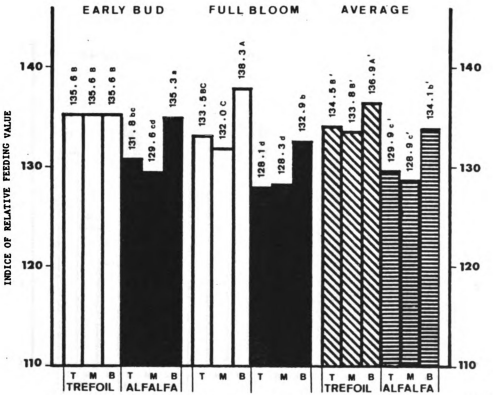


VERTICAL LAYERS T: top (1-6th nodes) M: middle (7-12th nodes) B: bottom (13-18th nodes)

Fig. 35 Vertical distribution of cell wall crude protein in stem of both alfalfa and birdsfoot trefoil at two maturity stages (Exp. II).

Comparisons between layers and maturity stages are shown by capital letter within trefoil and by small letter within alfalfa and the means with the same letter are not different at the 5 percent level by the Duncan's Multiple Range test.

Comparisons between layers of the bulk data within each species are shown by capital letter with ' for trefoil and by small letters with ' for alfalfa.



VERTICAL LAYERS T: top (1-6th nodes) M: middle (7-12th nodes) B: bottom (13-18th nodes)

Fig. 36. Vertical changes in the leaf indice of relative feeding value of both alfalfa and birdsfoot trefoil at two maturity stages (Exp. II).

Comparisons between layers and maturity stages are shown by capital letter within trefoil and by small letter within alfalfa and the means with the same letter are not different at the 5 percent level by the Duncan's Multiple Range test.

Comparisons between layers of the bulk data within each species are shown by capital letter with ' for trefoil and by small letters with ' for alfalfa.

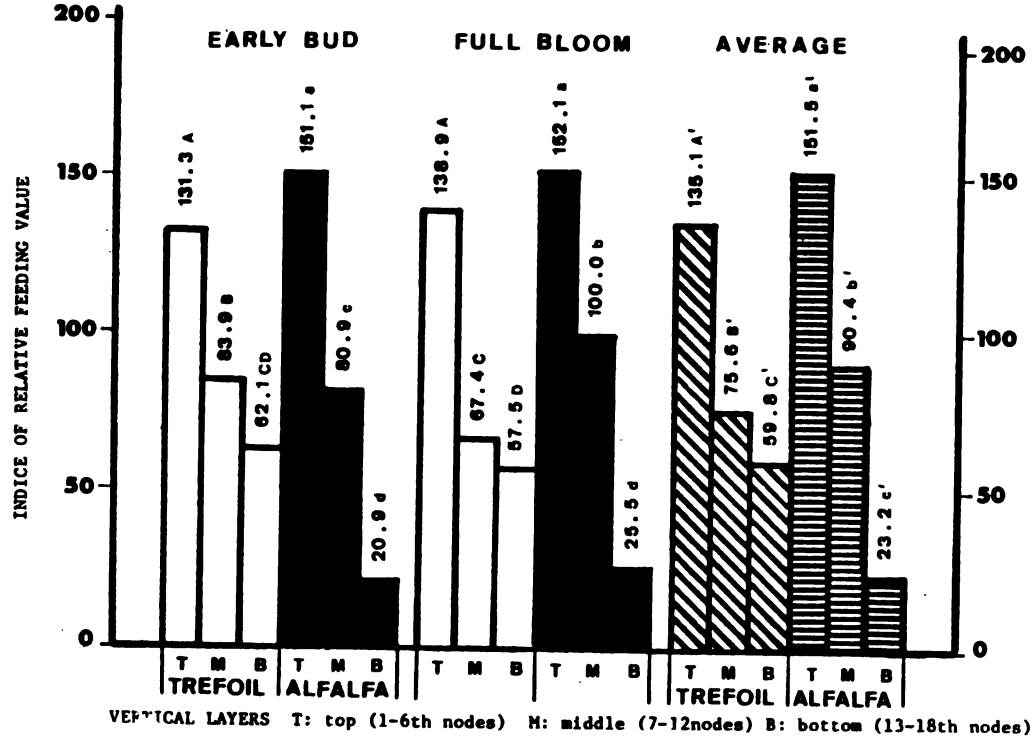


Fig. 37 Vertical changes of the stem relative feeding value of both alfalfa and birdsfoot trefoil at two stages of maturity (Exp.II).

Comparisons between layers and maturity stages are shown by capital letter within trefoil and by small letter within alfalfa and the means with the same letter are not different at the 5 percent level by the Duncan's Multiple Range test.

Comparisons between layers of the bulk data within each species are shown by capital letter with ' for trefoil and by small letters with ' for alfalfa.

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CHAPTER IV

DIGESTIBILITY STUDY
OF
ALFALFA AND BIDSFOOT TREFOIL

ABSTRACT

Alfalfa (*Medicago sativa L.*) and birdsfoot trefoil (*Lotus corniculatus L.*),grown in a greenhouse, were collected at early bud and at full bloom to determine the variation of IVDMD among three groups of plant development for each species and to compare the vertical and horizontal changes of IVDMD of each plant part.

The IVDMD of the three groups (1: 10-14 nodes; 2: 18 nodes; 3: 24-28 nodes) within each growth stage did not differ at $p < 0.05$. Species and the stage of maturity did not have a significant effect on the forage IVDMD. Fermentation for 24 hours in the rumen was adequate to evaluate the IVDMD of both alfalfa and trefoil.

Leaves and stems were collected from plants with 18 nodes. Leaf digestibility of alfalfa was higher than trefoil at every maturity stage. Alfalfa leaves was more digestible than trefoil at the two upper layers. Digestibility increased between 12 and 24 hours but was not affected by stages of maturity and position on the stem.

Stem digestibility was affected by the position on the shoot rather than by species, maturity stages and fermentation time.

The IVDMD of the stem decreased from plant top to bottom within alfalfa but trefoil showed some discrepencies in the pattern of changes of the stem IVDMD.

INTRODUCTION

Feeding value of forage species for ruminants can be evaluated by an *in vivo* or *in vitro* rumen fermentation. The two stage rumen fermentation *in vitro* proposed by Tilley and Terry (1963) and modified by both Goering and Van Soest (1970) and Marten and Barnes (1979) is considered the most reliable technique for the estimation of digestibility *in vivo* (Barnes 1966; Ademosum et al, 1968; Mayer et al, 1971; Nelson et al, 1975; and Horton et al, 1980). For this reason the *in vitro* procedure of Tilley and Terry was chosen for the present study.

The two stage technique *in vitro* measurement of forage digestibility attempts to estimate the digestible soluble fraction of forage as well as the digestible fibrous fraction. The first stage involves substrate fermentation by rumen microorganisms incubated for several hours. A period of 24 hours of incubation is considered adequate to estimate the *in vivo* digestibility of legumes (Hopson et al, 1963; Donefer 1970; Reid et al, 1960; and Chenost et al, 1966).

The second stage is the digestion of the first stage residue with hydrochloric acid-pepsin for 48 hours. Marten and Barnes (1979) pointed out that this step was used to simulate the *in vitro* breakdown of feed and microbial protein by the digestible enzymes of the ruminant abomasum.

Using Tilley-Terry's *in vitro* procedure, several authors reported a decrease in digestibility of the whole alfalfa plant and birdsfoot trefoil, with advancing maturity (Horrocks and Washko,

1968; Barnes and Gordon, 1972; Hoveland and Monson, 1980; Wilman and Altimimi, 1984; Buxton et al, 1985). The latter trend was found to be related to the increase in the whole plants structural constituents (Allinson et al, 1969). In addition, Walters et al (1967) suggested that aging plants showed less digestibility because the herbage contained a higher proportion of senescent and dead material. When the digestibility of alfalfa was compared to birdsfoot trefoil during maturation, Anderson et al (1973) reported a faster decline of alfalfa digestibility than trefoil. A larger canopy mass of trefoil over that of alfalfa in a late growth stage was suspected to be partially responsible for the difference in digestibility between species.

The high dry matter digestibility of leaf remained relatively constant during plant maturation whereas the stem digestibility decreased (Terry and Tilley, 1964; Mowat et al, 1965; Chenost et al, 1970; Thom, 1978). Some authors reported that the digestibility of the plant parts, like the whole plants, frequently showed a differential response in concentration among the species (Wilman and Altimimi, 1984; Buxton et al, 1985). According to Buxton et al, the digestibility of birdsfoot trefoil leaf was often significantly inferior to that of alfalfa. Conversely, the stem digestibility of trefoil was greater than alfalfa at a mature stage. Buxton et al (1985) related the difference in stem digestibility between trefoil and alfalfa to the greater amount of younger tissue in the stem fraction of trefoil.

Within a particular forage species at a given stage of maturity, the digestibility of its plant parts may change vertically along the shoot. Terry and Tilley (1964), Christian et al, (1970), Thom (1978), Wilman and Altimimi (1984), and Buxton et al, (1985) reported that the leaves of alfalfa showed little change in digestibility with their position on the shoot whereas the stem digestibility progressively declined from top to bottom. Little information is available on the vertical changes in digestibility of birdsfoot trefoil. The study of Buxton et al pointed out that birdsfoot trefoil seemed to follow a similar trend of digestibility as that of alfalfa.

The purpose of the present study was to determine 1) if there was a variation in the digestibility of the dry matter of various segments within the same species at the same growth stage, 2) the rate of digestibility of those segments with maturity, and 3) and compare the dry matter digestibility of segments of alfalfa and birdsfoot trefoil vertically down the shoot and with maturity.

METHODOLOGY

Two separate trials were performed on alfalfa and birdsfoot trefoil 1) to evaluate the degree of variation in dry matter digestibility between the segments of each species for the same stage of maturity and 2) to compare vertically and horizontally the dry matter digestibility of each segment of both leguminous species.

EXPERIMENT I

The forage was collected at early bud and full bloom. The forage was separated into three groups: a) group 1, plants with few nodes. Because of the limited amount of material for this group with 10 nodes, a subsample of equal weight was taken from each group of plants with 10, 12 and 14 nodes and pooled to constitute the sample; b) group 2, plants with an intermediate number of nodes (18); and c) group 3, plants with a high number of nodes. The limited amount of material among the plants with 24, 26 and 28 nodes led to a pool subsample similar to group one of the same species. The IVDMD test was done on the bulked sample.

In vitro dry matter digestibility (IVDMD) was determined according to a modified two stage technique of Tilley and Terry (1963) amended by Marten and Barnes (1979). A 250 mg sample finely ground through a fine screen (1 mm) of a cyclone mill and transferred into a 50 ml centrifuge tubes. Twenty ml of buffer was placed into the tubes to which was placed into the tubes to which was added 5 ml of rumen fluid preparation (as described in Chapter V). Incubation took place in a water bath with the temperature maintained at 39^{o} C for 12, 24 and 48 hours.

After each fermentation, the buffer inoculum solution was re-

placed by a pepsin-hydrochloride acid solution and incubated for 48 hours in a water bath maintained at 39° C. The residue was recovered into a tared filter paper Whatman no. 54. It represented the indigested dry matter.

EXPERIMENT II

The material was prepared as previously described in Chapter III. The period of fermentation were 12 and 24 hours.

A 2 x 3 x 2 x 3 factorieal analysis of variance with two replications was used for trial 2 of each plant part. A Duncan's Multiple Range test was used to compare the mean difference between treatments within species and maturity stages. A t-test was performed on the mean of each group studied to determine possible differences between species.

RESULTS AND DISCUSSION

EXPERIMENT I

Fermentation time was the only variable that statistically affected the forage IVDMD. Plant development, species and maturity stage did not affect the IVDMD of the material. In addition, no interaction of the combined factors was statistically significant at $p < 0.05$ (Table 38).

Fermentation time: The forage IVDMD of both species averaged 51% dry matter, after a 12 hours fermentation period (Table 39). The rate of digestion of both legumes increased sharply between 12 and 24 hours of fermentation, from 51 to 64% of dry matter and then remained unchanged (67%) (Table 39).

The change of IVDMD with the time of incubation was also noticed in each species at both early bud and full bloom stages. Thus, 48 hours of fermentation was generally not needed to estimate the IVDMD of both legumes. This decision was in full agreement with other studies such as Reid et al (1960), Hopson et al (1963), Chenost et al (1966) and Allinson et al (1969).

Comparison between species: Alfalfa and birdsfoot trefoil averaged a comparable mean IVDMD with 61 and 57% dry matter, respectively (Table 39). A similarity in the forage digestibility of the whole plant alfalfa and birdsfoot trefoil was also reported by Ingalls et al (1965), Seaney and Henson (1970) and Collins (1983). This experiment suggests that the digestibility of the whole plant was conditioned by the proportion of the stem fraction as well as of its digestibility, as reported by Terry and Tilley (1964).

Table 38. Analysis of variance on the effects of factors species (S), maturity stages (M), plant development (P), plant layers (L) and fermentation time (F) on the digestibility of forage legumes in experiment 1 and 2.

Sources	Experiment 1	Experiment 2	
		Leaf	Stem
Species (S)	N.S.	**	N.S.
Stade of maturity (M)	N.S.	N.S.	N.S.
Plant development (P)	N.S.	-	-
Plant layers (L)	-	N.S.	**
Fermentation time (F)	**	**	N.S.
S X M	N.S.	N.S.	N.S.
S X P	N.S.	-	-
S X L	-	N.S.	N.S.
S X F	N.S.	N.S.	N.S.
M X P	N.S.	-	-
M X L	-	N.S.	N.S.
M X F	N.S.	N.S.	N.S.
P X F	N.S.	-	-
L X F	-	N.S.	N.S.
S X M X P	N.S.	-	-
S X M X L	-	N.S.	N.S.
M X P X F	N.S.	-	-
M X L X F	-	N.S.	N.S.
S X M X P X F	N.S.	-	-
S X M X L X F	-	N.S.	N.S.

The significance of factors was evaluated at 1 percent level and followed by ** and by * at 5 percent level and N.S. followed those which are not significant.

Table 39. Changes in *in vitro* dry matter digestibility (IVDMD) of different groups of plant development at three different fermentation time, within early bud and full bloom of both alfalfa and birdsfoot trefoil, in experiment I.

Species	Groups	Maturity Stages									
		Early Bud			Full Bloom						
		Fermentation time (hours)			Fermentation time (hours)			Average (hours)			
		12	24	48	12	24	48	12	24	48	
Alfalfa	Group 1 (10-12-14 nodes)	52 b	67 a	66 a	55 b	56 b	69 a	53 c	61 b	67 ab	
	Group 2 (18 nodes)	50 c	63 a	66 a	55 b	64 a *	65 a	52 c	63 ab	65 ab	
	Group 3 (24-26-28 nodes)	53 b *	67 a	69 a	52 b *	65 a	67 a	52 c	66 ab	68 a	
	Average	52 B	66 A	67 A	54 B	62 A	67 A	52 C	64 A	67 A	/ 61
Trefoil	Group 1 (10-12-14)	50 b	63 a	64 a	48 b	59 a	63 a	49 b	61 a	63 a	
	Group 2 (18 nodes)	47 b	64 a	66 a	52 b	57 ab *	58 ab	49 b	60 a	62 a	
	Group 3 (24-26-28)	50 b *	60 a	68 a	49 b *	58 a	63 a	49 b	59 a	65 a	
	Average	49 B	62 A	66 A	50 B	58 A	61 A	49 B	60 A	63 A	/ 57

- Comparisons between plant layers at both fermentation times and at the same maturity stage within each species, are shown by small letters and the means with the same letter are not significantly different at the 5 percent level by the Duncan's Multiple Range test.
- Comparisons between fermentation time for the same stage of maturity are shown by capital letters and the means with the same letter are not significantly different at the 5 percent level by the Duncan's test.
- Comparisons between plant layers and between both species at the same fermentation time are done by the t-test and the means that showed difference at the 5 percent level are followed by * .

In addition, the morphological composition may also exert an important influence on the digestibility of alfalfa and trefoil. The latter factor became very important in the nutritive value of the two legumes, since a difference in digestibility of leaves of both species has been demonstrated in Experiment II of the present chapter. Following this reasoning, it is understandable that full bloom alfalfa had a higher digestibility (65%) than full bloom trefoil (58%) (Table 39) because of its greater leaf-stem ratio as well as of its higher leaf digestibility. When the leafiness and the leaf digestibility are combined to give superiority of one species over another, the feeding value elements of a forage counterbalanced the influence of the stem fraction upon the overall forage digestibility (Chapter I, pp.13-18).

EXPERIMENT II

Leaf digestibility: The species as well as the fermentation period were the factors that statistically affected the level of leaf digestibility. The other factors such as stages of maturity and leaf position on the shoot did not play any important role in the digestibility of the leguminous leaves. No interactions between the factors were found significant (Table 38).

Species: Alfalfa averaged a leaf digestibility significantly greater (81%) than that of trefoil (73%) (Table 40). The latter trend between both alfalfa and trefoil occurred at each maturity stage (Table 40). Some chemical or anatomical features specific to each specie could be involved in the difference of leaf digestibility between alfalfa and birdsfoot trefoil.

Table 40. Changes in *in vitro* dry matter digestibility (IVDMD) of leaf, vertically down the shoot of both alfalfa and birdsfoot trefoil at two growth stages and at two fermentation time, in experiment II.

Species	Plant Layers	Maturity Stages: Early Bud, Fermentation time (hours) 12	Early Bud, 24	Full Bloom, 12	Full Bloom, 24	Average (hours) 12	Average (hours) 24
Alfalfa	Top (1-6 nodes)	77 a	** 87 a	* 77 cd	84 ab	* 77 c	* 85 a
	Middle (7-12 nodes)	82 a	86 a	76 d	* 87 a	* 79 bc	* 86 a
	Bottom (13-18 nodes)	78 a	88 a	77 cd	81 bc	* 77 c	84 ab
	Average	** $\overline{79}$ B	** $\overline{87}$ A	** $\overline{77}$ B	$\overline{84}$ A	** $\overline{78}$ B	** $\overline{85}$ A / 81 **
Trefoil	Top (1-6 nodes)	66 b	** 71 b	* 67 b	81 a	* 66 b	* 75 a
	Middle (7-12 nodes)	69 b	73 ab	70 b	* 80 a	* 69 b	* 76 a
	Bottom (13-18 nodes)	67 b	79 a	67 b	82 a	* 67 b	80 a
	Average	** $\overline{67}$ B	** $\overline{74}$ A	** $\overline{68}$ B	$\overline{81}$ A	** $\overline{67}$ B	** $\overline{77}$ A / 72 **

- Comparisons between plant layers at both fermentation times and at the same maturity stage within each species, are shown by small letters and the means with the same letter are not significantly different at the 5 percent level by the Duncan's Multiple Range test.
- Comparison between fermentation time for the same stage of maturity are shown by capital letters and the means with the same letter are not significantly different at the 5 percent level by the Duncan's test.
- Comparison between plant layers and between both species at the same fermentation time are done by the t-test and the means that showed difference at the 1 percent level are followed by ** and by * at the 5 percent level.

Jones and Lyttleton (1971), Howarth et al (1978) and Lees et al (1982) reported the occurrence of tannin vesicules in the mesophyll cells of birdsfoot trefoil while none of these structures were observed in the alfalfa leaf (Gutek et al, 1974; Sarkar and Howarth, 1976; Goplen and Howarth, 1977; and Rumbaugh 1979). Van Sumere et al (1975) and Sarkar et al (1976) showed that condensed tannins inhibited cellulase and pectinase, limiting the leaf digestion by the rumen microorganisms. On the other hand, Howarth et al (1978) suggested that the mesophyll cells of a bloat-safe birdsfoot trefoil had cell walls with greater resistance to initial rupture by mechanical or rumen microrganisms than the mesophyll cell walls of a bloat-causing alfalfa.

Fermentation time: The leaf digestibility of both legumes was affected by the time of fermentation. A rumen fermentation of 12 hours gave a leaf IVDMD of 73% while 24 hours gave a IVDMD of 81%. Leaf digestibility increased 8% for alfalfa and 15% for trefoil between 12 and 24 hours of fermentation (Table 40).

Comparison of leaf digestibility between species: Some significant differences in the leaf IVDMD occurred between species. The mean IVDMD of the upper and median alfalfa leaves averaged 85 and 86% as compared to 75 and 76% of trefoil (Table 40).

Alfalfa had a greater proportion of secondary-tertiary leaves than trefoil at the two upper layers at both growth stages (Table 40). These young leaves may have increased the digestibility of the alfalfa leaf of these two upper segments. The limited digestibility of the upper layer of trefoil leaf might result from the presence of tannin in

the vesicules in the mesophyll cells. It was in early bud stage that the digestibility of the trefoil upper leaves was the most affected.

Early bud trefoil leaves showed a gradual increase in digestibility from the top (71%) to the bottom of the plant (79%) (Table 40). Such an observation led to two hypothesis based on Cutler's (1978) potential function of tannin. The tannin in younger leaves might act as an ultraviolet light shield, preventing possible damage to the chloroplasts, or this polyphenolic compound could be regarded as an astringent agent that protects the young leaves from being eaten by insects.

Median leaves of full bloom alfalfa had a higher IVDMD (87%) than those of trefoil (80%) (Table 40). Alfalfa had four times more easily degradable secondary-tertiary leaves than trefoil (Table 40).

Stem digestibility: The IVDMD of the stem was affected by the position of the segment on the shoot. Other factors such as species, maturity stages and fermentation time did not have a statistical influence on the stem IVDMD (Table 38).

Vertical layers: Alfalfa and trefoil declined from 75 to 48% in their stem IVDMD (24 hours) from top to bottom (Table 41), supporting the results of Terry and Tilley (1964) and Thom (1978) on alfalfa. They suggested that the decrease of stem IVDMD downward corresponded to the increase in structural material in the stem. In Chapter II (Table 14), the amount of supporting tissues such as sclerenchyma and xylem was significantly greater at the two lower layers (middle and bottom) than at the upper segments. The increased percentage of the above tissues downward in the shoot was more consistent in alfalfa than in trefoil.

Table 41. Changes in *in vitro* dry matter digestibility (IVDMD) of stem, vertically down the shoot of both alfalfa and birdsfoot trefoil, at two growth stages, and at two fermentation time, in experiment II.

Species	Plant Layers	Maturity Stages: Early Bud, Fermentation time (hours) 12	Early Bud, 24	Full Bloom, 12	Full Bloom, 24	Average (hours) 12	Average (hours) 24
Alfalfa	Top (1-6 nodes)	78 a	80 a	* 82 a	* 83 a	79 a	* 82 a
	Middle (7-12 nodes)	57 bc	63 b	56 b	59 b	56 b	61 b
	Bottom (13-18 nodes)	46 c	50 c	42 d	46 c	44 c	48 c
	Average	$\overline{60}$ A	$\overline{64}$ A	$\overline{60}$ A	$\overline{63}$ A	$\overline{60}$ A	$\overline{64}$ A / 62
Trefoil	Top (1-6 nodes)	59 b	74 a	* 60 ab	* 65 a	60 b	* 69 a
	Middle (7-12 nodes)	53 bc	56 bc	53 bc	57 ab	52 d	56 c
	Bottom (13-18 nodes)	48 c	51 bc	46 c	48 c	46 f	49 e
	Average	$\overline{53}$ A	$\overline{60}$ A	$\overline{57}$ A	$\overline{57}$ A	$\overline{53}$ A	$\overline{58}$ A / 56

- Comparisons between plant layers at both fermentation times and at the same maturity stage within each species, are shown by small letters and the means with the same letter are not significantly different at the 5 percent level by the Duncan's Multiple Range test.
- Comparisons between fermentation time for the same stage of maturity are shown by capital letters and the means with the same letter are not significantly different at the 5 percent level by the Duncan's test.
- Comparisons between plant layers and between both species at the same fermentation time are done by the t-test and the means that showed difference at the 5 percent level are followed by * .

The IVDMD (24 hours) of the upper stem of trefoil was greater (74 and 65%) than that of the basal stem (51 and 48%) at both stages and the median stem (56%) at the early bud stage (Table 41). Full bloom plants were similar in stem IVDMD at the top (65%) and middle (57%) layers. The occurrence of branches at the median and basal layers of early bud trefoil did not exert a positive influence in stem digestibility of corresponding layers but the inflorescences affected the digestibility at the upper and median layers of full bloom plants.

Comparison of stem digestibility: Stem IVDMD of both legumes within each layer and each maturity stage was similar except in full bloom plants where the upper stem of alfalfa had a higher IVDMD (83%) than trefoil (69%) (Table 41). Inflorescences composed 56% of the upper stem in alfalfa and 46% in trefoil (Table 42) which were found to have nutritive value close to leaves (Woodman and Evans 1935).

Comparison of the leaf and stem digestibility: With 24 hours fermentation, leaves were 30% higher than stems in IVDMD (Table 41). The difference in digestibility between leaf and stem of both legumes increased down the shoot from a similar IVDMD at the top layer to a difference 40% in favor of the leaves at the middle layer and 67% at the bottom segment. Species reacted similarly except that the difference in IVDMD between leaf and stem was greater in alfalfa.

Table 42. Contribution (%) of inflorescence to the total stem fraction of each layer on both alfalfa and birsfoot trefoil at every maturity stage (Exp. II).

Species	Layers	Maturity Stages			
		Early Bud (%)	10% Bloom (%)	Full Bloom (%)	Average (%)
Alfalfa	Top (1-6 nodes)	0	33	56	30
	Middle (7-12 nodes)	0	0	4	1
	Bottom (13-18 nodes)	0	0	0	0
	Average	0	11	20	10
Trefoil	Top (1-6 nodes)	0	40	46	29
	Middle (7-12 nodes)	0	1	8	3
	Bottom (13-18 nodes)	0	0	0	0
	Average	0	14	18	11
Average		0	12	19	—

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CHAPTER V

RUMINAL DEGRADATION OF LEAF AND STEM OF ALFALFA AND BIRDSFOOT TREFOIL BY ELECTRON MICROSCOPY

ABSTRACT

Leaves and stems were collected from alfalfa (*Medicago sativa L.*) and birdsfoot trefoil (*Lotus corniculatus L.*) at top, middle and bottom of of plants at early bud, 10% bloom and full bloom. Three aspects were studied: a) the ultrastructure of leaf and stem from various position along the shoot exposed to a ruminal fermentation; b) the pattern of degradation of the various tissue types of both legumes with maturity; c) the interrelationships between the rumen bacteria and the various tissue types.

Digestion of most tissue types in the leaf of both legumes was partial after 6 or 12 hours of fermentation. Mesophyll cells and the phloem showed visible degradation after 12 or 24 hours, at every position and at every maturity stage.

Digestion of the leaf and stem of both legumes resulted in some bacterial events characteristized by a) the types of anatomical components attacked by the bacteria; b) the surface potential of the tissue; c) the types of microorganisms in the ruminal inoculum; d) the interrelationships between bacteria on a particular substratum; and e) the interaction between the bacteria and the substratum .

INTRODUCTION

The use of scanning electron microscope (SEM) has been a useful tool to explore the microanatomy of a specimen since it provides a highly magnified three dimensional image with a great depth of focus. This technique has been used to investigate rumen microorganisms degradation of structural components by Akin et al (1973) on tall fescue and coastal bermudagrass, Brazle et al (1979) on bluestem, Brazle and Harbers (1979) on alfalfa, Harbers and Thouvenelle (1980) on corn and sorghum, and Harbers et al (1981) on smooth bromegrass.

Akin and Burdick (1975) characterized the differential degradation of various tissues in leaves during the ruminal digestion as follows: the mesophyll cells and phloem are readily degraded, and lignified vascular tissues resist digestion. Consequently, the latter authors proposed that a forage with high amount of mesophyll and phloem would have a high digestibility.

In the study of Brazle and Harbers (1979) on the digestion of alfalfa leaves, mesophyll cells were partially to completely degraded and vascular tissues were partially digested with undegraded external structure after 24 hours of fermentation. Alfalfa stems showed only a partial disappearance of a dense zone of cells beneath the cuticle, following 24 hours of fermentation. After 48 hours of fermentation, the latter zone of cells beneath the cuticle completely disappeared. Most of vascular tissues as well as cuticle was still undegraded after 72 hours of fermentation.

Akin et al (1977 a) reported that the digestion of the various anatomical components in leaves of coastal bermudagrass was affected by their position along the stem as well as by the growth stage. The mesophyll cells were digested to a lesser extent at the bottom than at the top portion of the leaf sheath. However, the bottom leaf blades were digested as much as the top leaf blades. In a recent paper, Akin et al (1984) found in *Panicum milioides* and in *Panicum laxum* that the mesophyll cells in bottom lamina tended to be more readily degraded than those in the upper lamina (even after 48 hours of fermentation).

The augmentation of lignification of parenchyma cells in stem limited their degradation with maturity on wheatgrass and bromegrass (Pigden, 1953), and on coastal bermudagrass (Akin et al, 1977 b).

In the process of digestion of the plant structural components, some interrelationship occurred between the rumen bacteria and the plant walls. Several authors (Akin and Amos, 1975, 1979; Akin, 1976 a, b, 1979; Amos and Akin, 1978; Harber and Thouvenelle, 1980; Brazle and Harbers, 1977) have reported attachement of some rumen microorganisms to plant tissues following observations with SEM. For instance, rumen bacteria like cocci appeared to need attachement to plant walls to degrade them (Akin et al, 1974; Akin and Amos, 1975). In addition, several bacilli also need to adhere to the substrate. Patterson et al (1975) found that the bacilli got attached to the substrate by using fibrous material surrounding the bacteria cell wall.

Akin et al (1974) reported a change in the physical relation-

ship between the rumen bacteria during the digestion and the plant tissue types. Thus, rumen bacteria appeared to adhere to the thick cell walls on the epidermis and of bundle sheath prior to degradation. However, bacteria attacking mesophyll cells and phloem showed no need of attachement (Akin et al, 1984). Rumen bacteria did not attach to the cuticle.

The rumen bacteria favored three different ways to attack plant cell walls (Moore and Mott, 1973 and Collings, 1979). Both authors reported that rumen microorganisms attacked the plant cell wall by 1) invasion of the middle lamella of adjoining cells; 2) penetration of the lumen of plant fibers; 3) invasion of the fractures which may result in the a) removal of the cuticle b) the mechanical breaks of fibers during the process of rumination, and c) the destruction of the internal structure leading to the collapse of the structure contributing to the increase of fractures.

In the present study, the objectives were 1) to examine the ultrastructure of leaf and stem from various position along the shoot of both alfalfa and birdsfoot trefoil exposed to a ruminal fermentation; 2) to compare the pattern of degradation of the various tissue types of both legumes with maturity; and 3) to examine the interrelationship between the rumen bacteria and the various tissue types.

METHODOLOGY

Alfalfa and birdsfoot trefoil were grown in the greenhouse as in Chapter I. Samples were collected as in Chapter II. Fresh samples were sectioned in 3 mm lengths at the midpoint of the leaflet and separated in two equal portions so as to have one portion in buffer and one segment in the rumen inoculum. Stem specimens were cut in 3 mm lengths just above the selected node on the plants with 18 nodes (Chapter II) and separated in two equal segments for the same purpose previously mentioned. The samples were placed on dry ice and stored in a deep freeze until used.

Samples were allowed to reached room temperature progressively. Sections of specimens of each plant part were transfered into polyethylene tubes (50 ml) containing Mc Dougall's buffer and rumen inoculum in a ratio of 1:4 and gassed with a stream of CO_2. A blank check was also used at each incubation time. Rumen fluid was collected from a fistulated Holstein cow fed twice daily on an alfalfa hay diet. Rumen fluid was collected, strained through four layers of cheesecloth and placed in a prewarmed thermos ($39^{o}C$). The ruminal fluid was centrifuged once at 1000 r.p.m. for one minute to sediment foreign matter and protoza. The surpernantant was centrifuged at 5000 r.p.m. for 20 minutes. The resulting residue suspended in a warm phosphate-carbonate buffer ($39^{o}C$) at pH 7.0 (as described by Cheng et al, 1955) served as a source of rumen bacteria inoculum.

After 6, 12, 18 and 24 hours of fermentation at $39^{o}C$, specimens were removed and fixed in 5% glutaraldehyde for several hours, washed

with a 0.1M cacodylate buffer at pH 7.0, and dehydrated in ethanol. Specimens were then subjected to a critical point drying procedure, mounted on aluminum stubs, coated with about 300 Å of gold in a film-vac sputter coater. Specimens were observed on a ISI-SUPER III SEM operated at 15 Kvolts. Photographs were on films negative-positive Polaroid 665.

RESULTS AND DISCUSSION

Leaf: Alfalfa and trefoil leaves from various positions on the stem showed partial degradation of the mesophyll cells and the phloem at the early incubation times. The epidermis and the vascular vessels were the site of bacterial activity but there was little visible evidence of degradation after 6 to 12 hours of fermentation (Fig. 38 and 39). With a 12 hours fermentation period, most of the above tissue types showed some degradation (Fig. 40 and 41).

The epidermal layer of the adaxial side of the alfalfa leaf was attacked randomly by dense populations of cocci in cluster (Fig. 42 and 43). These bacteria had appendages that could cause them to adhere to the tissue (Fig. 44). Subsequently, some rod-shaped bacteria invaded the area of the cocci where the epidermal surface was eroded (Fig. 45).

The degradation of epidermis cells appeared to occur after the rod-shaped bacteria were adhered on the tissue in a horizontal position (Fig. 45). The existence of ramifications that linked bacteria of the same species and/or of the different species together during digestion are shown in Fig. 45 and 46. The reasons for this type of association are unclear but it could be related to the need of the microorganism to be hold firmly to the tissue in order to be not easily removed.

The degradation of mesophyll cells of alfalfa leaf seemed to involve, besides the bacteria, some other microorganisms such as fungi (Fig. 47), in support of the report of Lund (1974). The need of attachment to the tissue was not a requirement for all species of bacteria acting in the digestion.

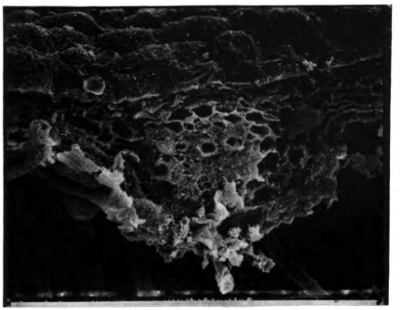

Figure 38. Leaf of alfalfa incubated 12 hours in a ruminal solution. X400.

Figure 39. Leaf of birdsfoot trefoil incubated 12 hours in ruminal solution. X400.

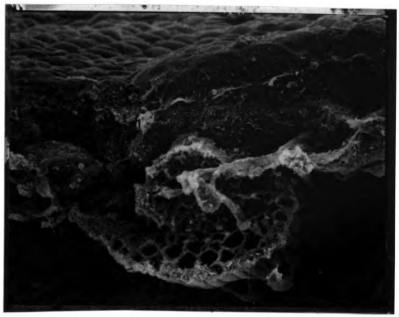

Figure 40. Leaf of alfalfa incubated 24 hours in a ruminal solution. X400.

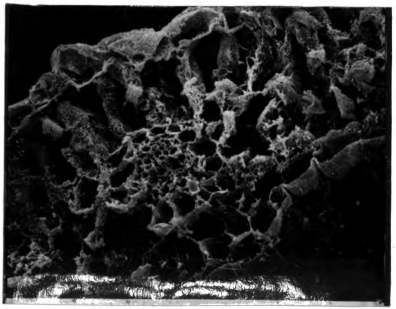

Figure 41. Leaf of birdsfoot trefoil incubated 24 hours in a ruminal solution. X400.

Figure 42. Colonies of cocci sp. eroding the epidermis of the alfalfa leaf after 12 hours of fermentation. X400.

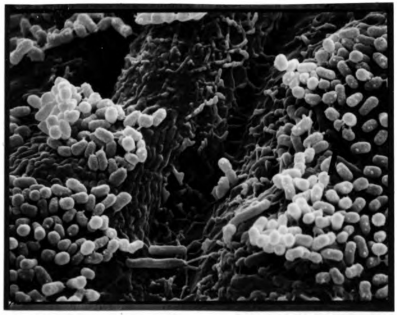

Figure 43. Magnified view of colonies of cocci sp. eroding the epidermis of the alfalfa leaf. The bacteria seemed to be linked together by some lateral appendages (arrow) (12 hours of fermentation). X5000.

Figure 44. The stomate is a location on the leaf epidermis where bacteria can penetrate to degrade the internal tissues (12 hours of fermentation). X5000.

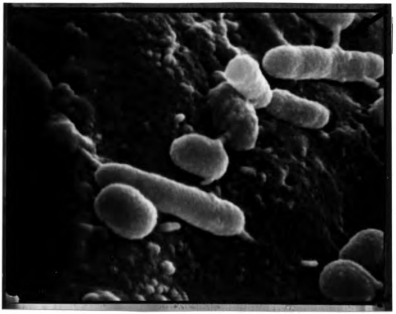

Figure 45. Bacteria with rod shape as well as with round shape showed attachement to the epidermis surface. They appeared to have some appendages that linked them (arrow) (12 hours of fermentation). X20000.

Figure 46. Population of ruminal bacteria degrading the epidermal cells in an eroded area of an alfalfa leaf. Bacteria showed ramifications between them which could be used to adhere to the tissue (24 hours of fermentation). X5000.

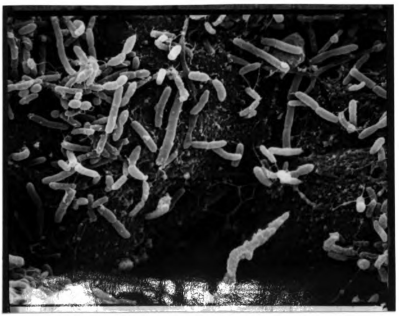

Figure 47. Population of ruminal bacteria interspersed with fungi (mycellium; arrow) on mesophyll cells of alfalfa leaf (12 hours of fermentation). X5000.

Several species of rod-shaped bacteria were involved in the digestion of phloem tissue in alfalfa leaf (Fig. 48). The bacteria attacked the periphery of the tissue at an angle without visible attachement. Round-shaped bodies on the cell wall of rod-shaped bacteria were frequently attached to cell wall of the bacteria.

Leaf vascular vessels such as xylem were attacked by the rod-shaped bacteria at the middle lamella (Fig. 49). The degradation of the middle lamella was at the periphery or at any exposed edges by bacteria. Consequently, the number of exposed edges that would be attacked by bacteria (specially rod-shaped bacteria) would likely determine the degree of degradation of the tissue. This is confirmed to some extent by Akin and Amos (1975) on the leaf of coastal bermudagrass and Brazle et al (1979) on bluestem. Some cellulolytic cocci species preferred the internal part of the xylem as location to degrade the cellulose (Fig. 49).

Bacteria randomly attacked the epidermal layer on leaves of birdsfoot trefoil (Fig. 50). The bacterial population seemed to be lower than that on alfalfa leaves. Fimbriae bacteria showed preferential growth on the epidermal layer of the trefoil leaf (Fig. 51). Meadows (1971) found that bacteria with pili adhered to surfaces having an electrostatic barrier. The high population of the bacteria on the leaf epidermis suggested that trefoil had a surface potential different than that on alfalfa.

The digestion of trefoil mesophyll cells (Fig. 52) was initially slower than that of alfalfa after 12 hours of fermentation. Little bacterial activity on the mesophyll cells of trefoil could explain this observation. However, the increase of the time of fermentation allowed

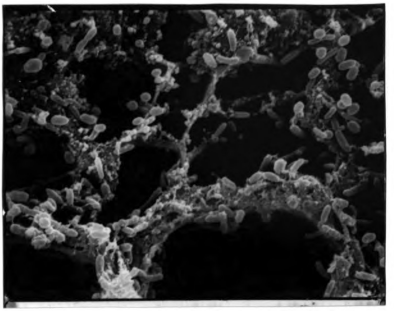

Figure 48. Phloem in the main vein of an alfalfa leaf attacked by rod bacteria sp.. Several rod bacteria showed round shape bodies along their cell wall. These bodies had appendages to permit them to adhere to the rod bacteria (12 hours of fermentation). X5000.

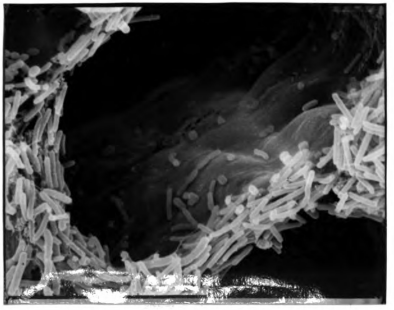

Figure 49. The main vein xylem of alfalfa leaf attacked by a dense population of bacteria of rod shape at the periphery and at the middle lamella of the tissue (24 hours of fermenattion). X5000.

Figure 50. Bacterial population on the epidermis of birdsfoot trefoil, after 24 hours of fermentation in a ruminal solution. X5000.

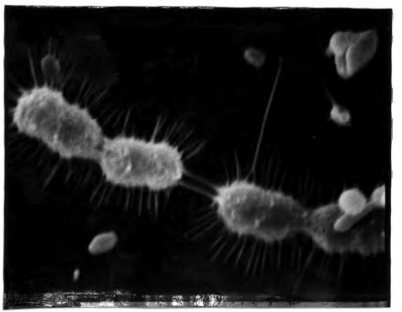

Figure 51. Fimbriae bacteria found in great numbers on the epidermis of the birdsfoot trefoil leaf (24 hours of fermentation. X20000.

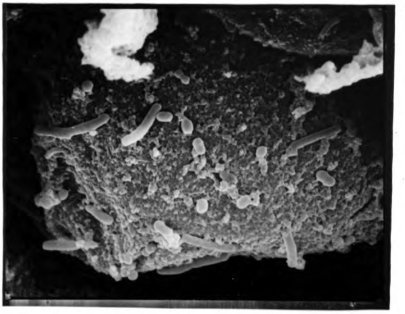

Figure 52. Mesophyll cells of birdsfoot trefoil leaf incubated 12 hours in a ruminal solution. X5000.

Figure 53. Mesophyll cells of birdsfoot trefoil incubated 24 hours in a ruminal solution. X5000.

bacteria to adhere densely to the tissue to digest it (Fig. 53).

The population of rod-shaped bacteria (Fig. 53) appeared to be more homogenous on mesophyll cells of trefoil than on of alfalfa Fig. 47). Very few ramifications linking bacteria together occurred on mesophyll cells of trefoil. The rod-shaped bacteria degraded the tissues along the cell wall of the microorganisms suggesting the occurrence of a slime around the bacteria cell wall containing hydrolitic enzymes. The latter structure also allowed the bacteria to firmly adhere on a horizontal position.

The digestion of the phloem and the xylem in the trefoil leaf followed a similar pattern as previously mentioned for alfalfa (Fig. 48 and 49).

Stem: The digestion of the stem of both legumes by the rumen bacteria followed a similar pattern as far as the degradation of the cortical parenchyma, phloem and at the partial degradation of the xylem, sclerenchyma and epidermis are concerned. The tissues in the upper stem of trefoil were digested to a greater extent (Fig. 54) than at the two lower stem segments (Fig. 55).

Degradation of some tissue types in the stem of both legumes was noticed after 6 hours of fermentation. Thus, the parenchyma cells in cortex and the phloem started to be digested, as showed in the top stem of 10% bloom trefoil (Fig. 56). The digestion of both tissue types were almost complete after 24 hours of fermentation (Fig. 57). The vascular vessels, the sclerenchyma and the epidermis showed little visible changes in their degradation from 6 (Fig. 56) to 24 hours (Fig. 57) of incubation. This was also obvious for the other stem segments.

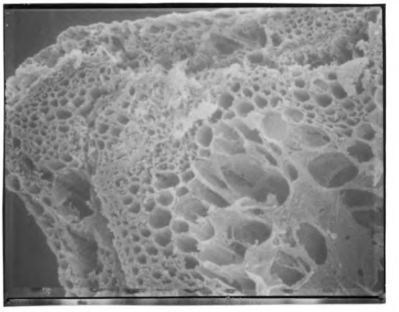

Figure 54. Birdsfoot trefoil stem collected at the top portion after a fermentation period of 24 hours in a ruminal solution. X400.

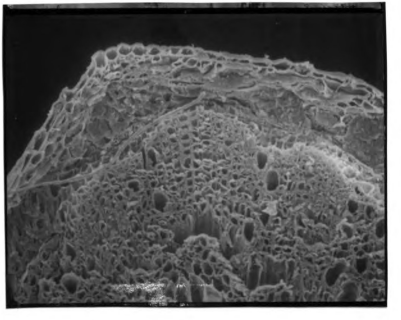

Figure 55. Birdsfoot trefoil stem collected at the bottom portion, after a fermentation of 24 hours in a ruminal solution. X400.

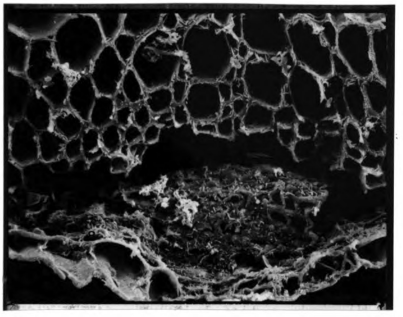

Figure 56. Trefoil stem incubated 6 hours in the rumen-buffer solution. X400.

Figure 57. Trefoil stem incubated 24 hours in a rumen-buffer solution. X400.

The stem epidermis, like the leaf epidermis, was randomly attacked by the bacteria. After 24 hours of fermentation, the stem epidermis was still intact (Fig. 57).

The cortical parenchyma and the phloem were rapidly degraded. Twenty-four hours of fermentation was enough to bring the tissue digestion behind the depth of focus (Fig. 57). The rod-shaped bacteria adhered tightly to the tissues before starting the digestion (Fig. 58).

The vascular vessels were not easy to degrade. They remained almost intact although a great bacterial activity was present during the 24 hours of fermentation period. Figure 59 shows bacteria of rod-shape attacking the middle lamella area of the upper stem trefoil. Some bacteria were held on the xylem cell wall by an appendage occurring at one end of its body cell wall (arrow). At the other extremity of the bacteria (Fig. 59) an extracellular substance that would contain hydrolytic enzymes would digest the structural polysaccharides because of the changes in the depth of focus around this substance (corresponding to zones of degradation (arrow)).

The above hydrolytic activity of the rod-shaped bacteria at the middle area of the cell wall could be needed so as to create breaches of the outer part of the cell walls. Consequently, the physical changes in the cell wall may allow cellulolytic bacteria to digest the cellulose layers located inside the cell wall (Fig. 60). The ease with which bacteria would attack and cause breaches in the outer layer of the cell wall would determine, to some extent, the degree of digestion of the conducting water tissues.

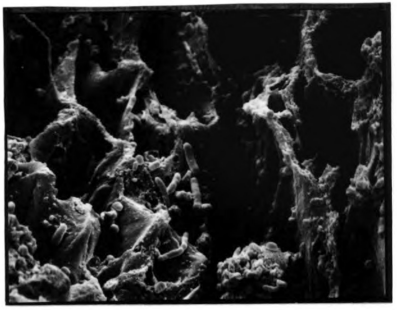

Figure 58. Bacterial activity on phloem of alfalfa stem after 6 hours of fermentation. The rod shape bacteria showed lateral link with other microrganisms of round shape. X5000.

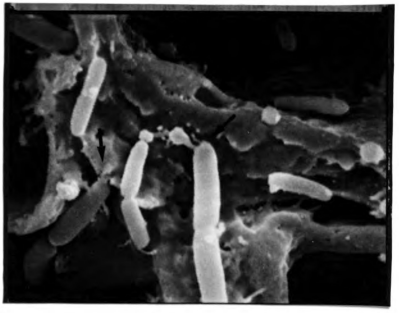

Figure 59. Bacteria of rod shape sp. degraded (arrow) the middle lamella area of the xylem cell wall of trefoil upper stem (24 hours of fermentation). X10000.

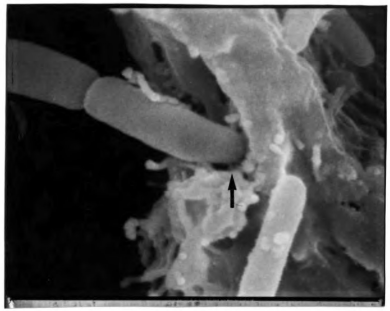

Figure 60. Cellulolytic bacteria attached to the internal wall of xylem in trefoil upper stem (24 hours of fermentation). X20000. The bacteria produced some bridging polymers that could be used to overcome the electrostatic repulsion barrier (arrow).

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