USE OF HISTOCHEMISTRY, SECTION-TO-SLIDE DIGESTION AND IMAGE ANALYSIS FOR EVALUATION OF DIGESTIBILITY IN REED CANARYGRASS (PHALARIS ARUNDINACEAE L.) GENOTYPES

A Thesis
Submitted to the Faculty
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by

Elizabeth C. Thomsett

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of
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ABSTRACT

Plant breeders have expressed a need for development of selection techniques that are simple, quick and inexpensive, and provide information to animal nutritionists and plant breeders. These experiments were designed to evaluate histochemistry, section-to-slide digestion and image analysis techniques for their usefulness in forage digestibility evaluation programs. Enzymatic dry matter digestibility (DMD), lignin, acid detergent fibre, p-coumaric and ferulic acids, and neutral sugar contents were also measured.

On the basis of a gravimetric digestibility technique, two low digestibility genotypes and two high digestibility genotypes from the 9 parental genotypes of the RCG cultivar Rival were chosen for further analysis.

The four genotypes were harvested for stem material at 3 stages of growth and evaluated for digestibility. Tissues examined were the epidermis (E), cortex (C), phloem (P), sclerenchyma ring (SR), xylem (X), vascular bundles (VB) and parenchyma (PA).

Histochemistry indicated strong genotype and growth stage differences with all tissues, except P and X. However, only the acid phloroglucinol staining of the SR and PA, and the C with both stains provided similar values between both low digestibility genotypes. Tissue area percentages indicated that the P, SR, and PA were different among genotypes, but only the SR had a maturity effect. The only tissues which had similar areas between the two low digestibility genotypes were the PA and SR. Cell wall length, while not different, did show expected trends, less digestible genotypes had greater

lengths of less digestible tissues and vice versa. Lignin and acid detergent fibre concentrations and section-to-slide digestion indicated genotype and growth stage differences, but did not provide similar values between the low digestibility genotypes. Phenolic acid and neutral sugar concentrations and enzymatic digestibility analyses did not indicate genotype or growth stage differences.

It was concluded that histochemistry and image analysis are of potential value to plant breeders. Secondly, both biochemical (lignin) and microanatomical (percent area of tissues) characteristics are important when evaluating digestibility of forage genotypes. The SR and PA may be the key tissues involved in digestibility differences among genotypes and growth stages.

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LIST OF ABBREVIATIONS

ADF	acid detergent fibre
ADL	acid detergent lignin
AP	acid phloroglucinol
C	cortex
°C	degrees celsius
cm	centimetre
CP	crude protein
CS	chlorine sulphite
cv	cultivar
CW	cell wall(s)
CWC	cell wall constituents
DM	dry matter
DMD	dry matter digestibility
E	epidermis
FA	ferulic acid
g	gram
HCL	hydrochloric acid
IA	image analysis
IVDMD	in vitro dry matter digestibility
kg	kilogram
ls	least squares
M	mesophyll
micron	micrometre
mg	milligram
ml	millilitre
N	nitrogen
NDF	neutral detergent fibre
NIR	near infrared
P	phloem
PA	parenchyma
PCA	p-coumaric acid
PBS	parenchyma bundle sheath
RCBD	randomized complete block design
RCG	reed canarygrass
RNA	ribonucleic acid
S	sclerenchyma
SEM	scanning electron microscopy
SR	sclerenchyma ring
STS	section-to-slide
umoles	micromoles
VB	vascular bundle

VI	voluntary intake
w/v	weight by volume
X	xylem

INTRODUCTION

Reed canarygrass (Phalaris arundinaceae L.) is a cool-season, high-yielding perennial grass with desirable environmental and agronomic characteristics (Miller 1984). The grass performs well under both hot, dry summer conditions and cooler, wetter fall conditions (Marten and Heath 1973; Smith 1981; Sheaffer and Barnes 1987). Reed canarygrass (RCG) has had limited inclusion in most areas due to low palatability caused by low digestibility and alkaloid content. Breeding to reduce alkaloid content has resulted in RCG genotypes free of tryptamines and beta-carbolines and with lower levels of gramine and hordenine (Woods and Clark 1971). Rapid reductions in digestibility with maturity in RCG is usually handled with careful grazing and cropping procedures (Smith 1981). With the renewed interest in RCG by plant breeders, the digestibility of RCG is of current concern.

Anatomical characteristics of a plant will affect the digestibility. Leaves usually have higher digestibilities than stems, with leaf sheaths having intermediate digestibilities (Norton 1982; Wilman and Altimimi 1982).

Biochemical components of forages also affect digestibility. Lignin is usually considered to be the major limitation to digestibility (Casler 1986). The cell wall content (mainly cellulose and hemicellulose) of forages also affect digestibility (Buxton 1989). Recently, phenolic acids have been linked to digestibility (Marten and Heath 1973; Burritt et al. 1982, 1984; Fahey and Jung 1990). Interactions among these compounds also will affect the digestibility of grasses (Morrison 1979; Cheeson 1983; Burritt et al. 1984).

Microstructure components of grasses, more recently, have been, considered in digestibility evaluations. Individual tissues of plants have varying digestibilities when incubated in rumen fluid (Akin and Burdick 1975; Akin 1979; Harbers et al. 1981; Akin 1989). Histochemical analysis of these tissues is often indicative of their digestibilities (Akin and Burdick 1981; Akin et al. 1984; Akin 1989). However, the majority of these experiments have been performed on leaves of warm-season grasses.

A major problem in forage grass improvement through breeding programs is the development of selection techniques which provide information relevant to both animal nutritionists and plant breeders (Coleman 1983). The techniques must be simple, rapid and relatively inexpensive, as plant breeders need to evaluate hundreds of genotypes of each forage species before implementing a breeding program for the grass (Coleman 1983). In vivo techniques, while providing the only truly accurate value for digestibility, are quite expensive, require large sample sizes and take several days for results. In vitro methods are less expensive but are also long and require fairly large sample sizes. Chemical methods, such as the detergent fibre system (Van Soest 1963), provide acceptable correlations with in vivo results, but the correlations are usually different for the different forage species; there is no one regression equation that works over all forage species (Burritt et al. 1984). Other techniques are being developed (image analysis) or improved from existing ones (histochemistry) which would assist plant breeders in selecting genotypes for inclusion in breeding programs.

The present study was undertaken to examine genotypes of the RCG cultivar Rival for digestibility using a modified in vitro gravimetric digestibility technique. Two high and

2 low digestibility genotypes from the 9 genotypes were selected for further analyses. Clones of the selected genotypes were grown for harvest at 3 maturity stages.

The acid detergent system of analysis was used to examine the percentages of ADF and lignin. A modified histochemical analysis with acid phloroglucinol and chlorine sulphite was used to detect differences in colour ratings, reflecting different amounts of coniferaldehyde and syringyl units of ligin and their location. A modified image analysis program was used to examine percent area, cell wall width and cell wall length of selected stem tissues. A modified section-to-slide technique was used for comparative examination of differences in stem tissue digestibility. Enzymatic and alkaline hydrolysis of ground stem material were performed to estimate stem phenolic acid and neutral sugar contents. A prepared enzyme incubation was used to examine genotype differences in dry matter digestibility.

The first objective was to identify differences among genotypes that may account for digestibility differences. Changes that occur with increased plant maturity were also examined. The results of these experiments were compared to each other in order to determine which methods closely relate to the others and thus may be of value to plant breeders.

LITERATURE REVIEW

CHARACTERISTICS OF REED CANARYGRASS

Agronomic Features

Reed canarygrass (Phalaris arundinaceae L.) is a cool-season, high-yielding, perennial grass with the C3 type of photosynthesis (Miller 1984). As a forage crop, reed canarygrass (RCG) has desirable environmental and agronomic characteristics. It is most commonly grown for hay or pasture as a monoculture crop, although RCG can also be used for silage (Miller 1984; Marten 1985). The height of RCG can reach between 60-240 cm (Miller 1984).

Reed canarygrass has excellent tolerance to wet conditions (Sheaffer and Barnes 1987) and grows best on fertile, low, moist land which is too wet for most other grasses. The grass can also withstand fairly heavy and prolonged flooding (Smith 1981). Reed canarygrass will also grow well on drier land that has good fertility and moisture-holding capacity (Smith 1981). Best growth occurs in soils with pH ranging from 4.9 - 8.2 (Marten 1985), but RCG will tolerate acid soils and fairly high salinity (Sheaffer and Barnes 1987). Notwithstanding its advantages on wet soils, RCG also has good tolerance to drought and can provide adequate pasture even during dry summers (Smith 1981; Miller 1984; Sheaffer and Barnes 1987). Reed canarygrass tolerates frequent harvests and has excellent resistance to cold and frost in most growing areas (Sheaffer and Barnes 1987).

A relatively rare environmental characteristic in most domestic grasses, but present in RCG is its sod-forming potential which increases soil coverage and soil-holding capacity due to the deep and fibrous root system. This characteristic makes RCG an excellent grass for erosion control, especially in waterways and ditches (Smith 1981). Sod formation also reduces compaction of wet soils caused by animals and farm machinery (Marten 1985). Another advantage of RCG is its ability to resist lodging (Miller 1984). Reed canarygrass has few insect or disease problems, and these can usually be controlled by appropriate management techniques (Wolf 1967; Marten and Heath 1973).

Despite its obvious advantages, RCG does have some potential disadvantages. One possible disadvantage of RCG is the high requirement for nitrogen (N) that is necessary to obtain good dry matter (DM) yields (Smith 1981). However, RCG has higher recovery of applied N than many other grasses so the extra N is usually more fully utilized (Marten et al. 1976). It is often difficult to establish RCG with legumes due to the highly competitive nature of sod-forming grasses like RCG; however, with appropriate management, alfalfa and some clovers can be grown with RCG (Miller 1984). Other agronomic disadvantages of RCG include difficulty in sowing seeds due to easily-shattering and fluffy seeds, poor seedling vigour (Sheaffer and Barnes 1987) and slow-to-establish seedlings (Smith 1981; Miller 1984). Problems involving the establishment of RCG via seeding can be reduced by using vegetative propagation, which is the placement of plant sections in a spreader and depositing them on well-disced soil (Miller 1984). Once established, RCG has early, heavy spring growth and continues to perform well

under both hot, dry summer conditions and cooler, wetter fall conditions (Marten and Heath 1973; Smith 1981; Miller 1984; Sheaffer and Barnes 1987). These growth traits make RCG an excellent choice for pasture.

As with other cool-season grasses, RCG has lower herbage production during the summer than in the fall or spring (Sheaffer and Barnes 1987). Reed canarygrass provides very high yields of dry matter and can handle, even requires, high stocking densities to ensure that the growth is always at a stage that is palatable. Marten and Donker (1968) reported that highest animal acceptance occurred when RCG was grazed at heights between 15 and 60 cm.

Persistence of grasses is an essential characteristic for economical production. In an experiment where data was gathered for three years, Marten and Hovin (1980) reported good persistence of RCG at 2, 3, or 4 cuttings per year. Bromegrass, orchardgrass and tall fescue were more sensitive to the number of cuttings. Smith et al. (1973) found that RCG, tall fescue and orchardgrass had better persistence when grown with alfalfa than did timothy or bromegrass, and suggested that the differences were due to the continuous tiller production of the former grasses. In general, RCG persistence is only reduced if the grazing or cutting interval is too short, which suggests that RCG is best when grown for hay or rotational grazing.

Nutritional Characteristics

General. The low digestibility and palatability of RCG, the latter of which is due to alkaloid content, have been a barrier to its wide acceptance. Reed canarygrass has a rapid decrease in digestibility with maturity due to a quick accumulation of fibre (Smith 1981), necessitating careful grazing and cropping procedures. Conversely, the palatability of RCG due to alkaloid content increases with age, since the alkaloid content decreases with maturity (Van Soest 1985). Alkaloids will be discussed in the next section. The palatability of RCG can be improved by adding extra N, as the N content of the grass increases with the extra N fertilization and this increase leads to an improvement in digestibility (Marten and Heath 1973). Reed canarygrass was recently classified as one of the most likely grasses to cause grass tetany due to low magnesium levels or to high ratios of potassium relative to calcium and magnesium levels (Sheaffer and Barnes 1987).

Nutritionally RCG is a worthy forage. Although the crude protein (CP) content varies with plant part, level of applied N, and maturity, it can be quite high in RCG, ranging between 7 and 30% (Marten 1985). Since RCG is efficient at extracting N from the soil, under identical conditions, RCG often has higher CP content than other cool-season grasses (Marten 1985). The CP content of RCG is as great or greater than that of orchardgrass, smooth bromegrass, tall fescue, meadow fescue, meadow foxtail, timothy and tall oat grass (Miller 1984). However, the CP content declines faster with increases in maturity in RCG than in other grasses (Marten and Hovin 1980). Time of harvest for

the first cut is often a trade-off between dry matter (DM) yield and CP content since, as RCG ages, DM yields increase while CP content decreases (Marten and Hovin 1980). Bonin and Tomlin (1968) found that delaying the first cut past the heading stage reduced the CP content, and thus recommended harvesting RCG between the boot stage and the heading stage in order to provide maximum DM yield without reducing CP yield.

Dry matter yield, CP content and in vitro dry matter digestibility (IVDMD) all respond to increased N fertilization in RCG. In fact, at high levels of N fertilization, RCG can have the same CP content as alfalfa at the same point in growth (Phillips et al. 1954; Barnes and Mott 1970; Krueger and Scholl 1970; Lawrence et al. 1971). Overall digestibility of RCG can be quite high, often similar to alfalfa, and digestible energy is usually equal or greater than that of other cool-season perennial grasses (Marten 1985).

Other nutritional parameters are also important when comparing forages. The cell wall content (CWC), as measured by neutral detergent fibre (NDF), has been negatively correlated (-0.76) with voluntary DM intake (Van Soest 1965). Marten and Hovin (1980) reported that the CWC of RCG was less than that of tall fescue for 2 out of 3 years. The hemicellulose content of RCG is higher than that of alfalfa leading to a higher CWC (Donker et al. 1976). Acid detergent fibre (ADF) is negatively correlated with DM digestibility (Van Soest 1965). Despite higher values for ADF in RCG, no difference in DM digestibility between alfalfa and RCG were reported by Donker et al. (1976). This can be at least partially explained by the fact that the ADF lignin, referred to as acid-detergent lignin (ADL), is indigestible and is higher in alfalfa than RCG thus reducing the effect of the lower ADF in alfalfa. The further importance of these and other

nutritional components will be discussed later.

Alkaloids. One the major reasons for the relative lack of interest in RCG is alkaloid content. Since several recent theses (Duynisveld 1990; Tosi 1991) have dealt with RCG alkaloids, this section will only briefly introduce alkaloids and their importance in animal agriculture. Alkaloids are N-containing compounds with complex ring structures. Most researchers believe that alkaloids have no real function in plants since only 10 to 15% of vascular plant species contain alkaloids (Pelletier 1970), thus alkaloids are usually considered to be by-products of metabolism (Marten 1973). There are 9 known alkaloids in RCG: hordenine, gramine, 4 tryptamines and 3 beta-carbolines. Woods and Clark (1971) discovered RCG genotypes free of tryptamines and beta-carbolines and the authors suggested that a single dominant gene controlled the presence of these alkaloids. This discovery led to the production of genotypes and cultivars of RCG free of tryptamine alkaloids.

Tryptamine alkaloids are linked to "phalaris staggers" (incoordination, central nervous system disorders) and "sudden death" (collapse with death or occasionally total recovery, caused by ventricular fibrillation and/or cardiac arrest). Tryptamines may also affect the central nervous system by interfering with serotonin, a compound structurally similar to the tryptamines (Gallagher et al. 1964). Total alkaloid content in RCG has been reported to have a negative correlation (-0.95) with voluntary intake in sheep (Simons and Marten 1971). Alkaloid type and level were reported by Arnold et al. (1980) to affect intake and digestibility of RCG. Weight gains were also suggested to be affected by the type and level of alkaloids in RCG (Marten et al. 1976, 1981). Reed canarygrass has also been

suggested to cause watery eyes and rough haircoats in cattle (Van Arsdell et al. 1954). "Alkaloidal-type lesions" in the livers of dairy cows grazing RCG were reported along with reduced body weights and milk production (Audette et al. 1970). Coulman et al. (1977), however, reported that gramine, hordenine and two of the tryptamines did not affect IVDMD at the upper levels found in RCG. Arnold et al. (1980), however, reported that gramine at a level found in RCG inhibited the IVDMD of orchardgrass.

QUALITY OF GRASSES

For purposes of this paper, quality will be defined as the digestibility of the plant and the biochemical and physical components of plants influencing digestibility. The digestibility of grasses is influenced by many environmental and biological factors. Environmental factors, such as light intensity, photoperiod, temperature and water availability can affect both the growth and nutrient composition of grasses. Van Soest (1982) provides an excellent summary of the major environmental influences and their effects. Biological factors influencing digestibility include plant maturity, reproductive state of plant, disease or insect infestation and plant composition. Most books on forages provide sufficient detail on these factors. Wilson (1982) is an excellent starting point for interested readers. This section of the thesis will deal primarily with plant composition and the effects on digestibility. Plant composition can be described in terms of the biochemical constituents, anatomical characteristics and/or the specific microstructure of the plant. All three features will be discussed in this thesis.

Biochemical Components of Plants Affecting Digestibility

Cell Contents. For purposes of ruminant digestibility, plant cells can be divided into two parts, the cell contents or solubles, and the cell walls. The cell contents are very digestible and are potentially highly available to animals. Van Soest (1981) reported that cell contents are virtually 100% digestible and therefore are of little concern when considering quality, except that higher ratios of cell contents to cell walls provide for greater digestibility. Cell contents include proteins, sugars, starches, lipids and organic acids, and, unlike cell wall components, are not affected by lignification (Van Soest 1985). Glucose, fructose, sucrose, starch and fructosans are the major soluble carbohydrates in plants (Norton 1982).

Cell Walls. Cell walls (CW) of plants are defined as cellulose microfibrils embedded in a ligno-hemicellulose macromolecule to which acetyl and phenolic acid groups are bound (Morrison 1979). Bacon (1979) reported that in the initial development of the CW, the primary CW contain cellulose, hemicelluloses and pectin. During the secondary CW formation and thickening, lignin is deposited (Theander and Aman 1984). Cell walls represent 30-80% of plant DM.

The CW consists of potentially digestible and indigestible residues, therefore, the digestibility of the forage relates to the ratio of potentially digestible to indigestible CW material (Mertens and Ely 1982). Buxton (1989) stated that the digestibility of forages is limited by the CW concentration and the degradation of the CW.

Cell wall constituents (CWC) include phenolic acids, lignin, structural polysaccharides,

cutin, lignified nitrogenous substances, minerals, pectin, protein and waxes. The latter three compounds are soluble in the rumen (Van Soest 1982).

The "fibre" fraction of forages is defined as the cellulose, hemicelluloses and pectin content (Akin 1982a). Cellulose, one of the most abundant CW carbohydrates (CHO), consists of linear arrangements of D-glucose with beta (1-4) linkages and is insoluble in the rumen (Robinson 1983). Hemicelluloses were originally thought to be intermediates in cellulose formation, but actually are composed of a variety of polysaccharides, of which xylans make up the majority in most plants (Bacic et al. 1988). There are several different types of xylans (Robinson 1983). For purposes of this paper, the hemicelluloses will still be referred to simply as hemicellulose since most authors still use this term. Hemicellulose is the next abundant CW CHO after cellulose, and this compound, as well as cellulose, often occurs in conjunction with lignin, which is completely indigestible (Robinson 1983; Miller 1984). Burritt et al. (1982) found a fairly high negative correlation (-0.77) between percent xylose and IVDMD which they suggest is due to an increased association of lignin with hemicellulose xylans in the CW, thus resulting in reduced digestibility. A high negative correlation (-0.85) was reported by Burritt et al. (1984) for percent xylose (precursor of xylans) and IVDMD. Xylans were reported by Waite et al. (1964) to have a greater reduction in digestibility with maturity of grasses than either cellulose or hemicellulose polymers.

Cutin, located on the leaf surface and with the CW components, also is often linked with lignin and will reduce the digestibility of cellulose and hemicellulose if linked with these compounds (Miller 1984). Pectic substances, usually found in primary cell walls

and as binding material between cells, are highly digestible by ruminants (Robinson 1983; Miller 1984).

Phenolics. Plant phenolics, which include lignin, are also important in digestibility analyses. Phenolics have been defined by Fahey and Jung (1990) as

> "... a large heterogeneous group of secondary metabolites which are ubiquitous in the plant kingdom."

A wide variety of structures exist ranging from a single aromatic ring in simple phenolics to complex substances, such as the tannins and lignin, and knowledge of the properties of phenolics vary with the specific compounds. This section will be concerned primarily with simple phenolic acids, focusing on p-coumaric acid (PCA) and ferulic acid (FA), both belonging to the family of cinnamic acids. These are the most abundant phenolic acids in forages (0.5 to 2.0% of total cell wall) (Fahey and Jung 1990) and have been widely studied. Because lignin is a major deterrent to digestion, it will be discussed in a separate section.

Concentrations of phenolic acids may vary with tissue type (Harris et al. 1980), plant part (Hartley 1972; Hartley and Haverkamp 1984), age (Jung et al. 1983; Burritt et al. 1984; Buxton and Russell 1988) or species (Fahey and Jung 1990). Environmental conditions are likely to affect the levels of simple phenolics in plants, since different conditions will influence the lignin content, but little is currently known as to the actual effects on simple phenolics (Fahey and Jung 1990). The concentrations of free phenolics, not bound to any other substance, are very low relative to bound phenolic levels (Newby et al. 1980; Jung et al. 1983).

The functions of phenolics in plants are generally unknown, if indeed they serve real purposes. The phenolics are the largest group of secondary plant metabolites. Originally they were thought to be inert end products of metabolism; however, radioactive precursors administered to plants showed plant phenolics to be metabolically active, and some precursors had very rapid turnover rates (Wong 1973). Phenolic compounds located in actively metabolizing plant tissues consist mainly of glycosides. Administered phenolic substances were converted to glycosides, and this conversion is suggested to be a detoxification mechanism, similar to gluronic acid conjugation of phenolic compounds in animals (Fahey and Jung 1990). Wong (1973) suggested that since the levels of phenolics are affected by temperature, light and season, and the levels can taper off to zero, it is likely that phenolics can be degraded by plants; however, research to date has shown little evidence of catabolism of phenolics in plants.

Many phenolics are biologically active in plants, animals and microorganisms, and can be either beneficial or toxic (Fahey and Jung 1990). Some of the toxic effects of phenolics include interference with the metabolism of vitamins and estrogens, as well as acting as carcinogens or hepatotoxins in animals (Singleton and Kratzer 1969; Singleton and Kratzer 1973; Singleton 1981). Phenolics may also interfere with various enzymes and enzyme systems in both plants and animals. Simple phenolics have been reported to inhibit mammalian enzymes in vitro (Milic et al. 1972; Van Sumere et al. 1975; Humphries 1980). For example, pancreatic RNAase activity was reduced with additions of ferulic, gallic, caffeic and syringic acids (Van Sumere et al. 1975). Some phenolics, such as tannins (Damaty and Hudson 1979; Couch et al. 1980) and simple phenolics

(McManus et al. 1981) can also bind with proteins and reduce their availability. Some phenolics also have anti-carcinogenic, hormonal and/or anticellular and immunosuppressive activities (Fahey and Jung 1990).

Simple phenolics can be metabolized by microbes, although there is variation in the intestinal bacteria and rumen microorganisms which are involved in the metabolism of phenolics and the ease with which the phenolics are metabolized (Fahey and Jung 1990).

Phenolics also have effects on forage digestion. Total phenolics have been negatively correlated with in vitro digestion (Burns and Cope 1974; Burns et al. 1976). Additions of FA and PCA have been reported to reduce cellulose digestion in vitro and are negatively correlated with IVDMD of grasses (Marten and Heath 1973). Burritt et al. (1982, 1984) reported high negative (-0.84 to -0.88) correlations between IVDMD and the ratio of PCA to FA for RCG. Burritt et al. (1984) also reported a high negative correlation (-0.86) between PCA and IVDMD but no significant correlation between FA and IVDMD. Other simple phenolics, such as caffeic and sinapic, have also been linked to reduced digestibility of forages (Fahey and Jung 1990).

Several experiments have shown specific effects on rumen microorganisms. Experiments by Chesson et al. (1982) with several phenolic acids and pure cultures of rumen bacteria indicated that PCA and FA produced the most severe growth depression, and that cellulolytic bacteria species were most sensitive to the phenolic acids. Free PCA was reported by Akin (1982b) to inhibit the growth and motility of cellulolytic and xylanolytic bacteria, and the author suggested that when PCA was released through degradation of forages, the PCA may inhibit further digestion. Ferulic acid was reported

to have less effect than PCA. Jung (1985) reported that PCA and FA did not significantly inhibit hemicellulose digestion, but did significantly inhibit cellulose digestion. The author also reported that PCA had greater inhibitory potential than FA. Varel and Jung (1986) reported that PCA additions caused a greater reduction in the rate of bacterial growth that FA. The evidence presented thus suggests that PCA is more involved in reducing forage digestibility than FA. Hartley (1972) had earlier recommended the use of the concentrations of PCA and FA as indicators of forage digestibility since the PCA:FA ratio was significantly correlated with vivo digestion in ryegrass.

Although Fahey and Jung (1990) suggested in their recent review that simple phenolics primarily depress the growth rate of bacteria, rather than affecting fungi and/or protozoa, Akin (1982b) reported that reduced motility of rumen protozoa occurred with additions of PCA and FA. The degradation of forages by rumen fungi was inhibited by PCA and FA additions (Akin and Rigsby 1985). It should be noted that these experiments utilized free phenolics and, as mentioned, there are very low concentrations of free phenolics in forages.

Highly lignified CW often result in slower rates of microbial colonization and attachment than less lignified walls (Akin and Barton 1983), and it is likely that these lignified cell walls also contain high levels of simple phenolics; thus, simple phenolics may likewise affect colonization and attachment. Varel and Jung (1986) reported that cinnamic and vanillin acids reduced the rate of attachment of a fibrolytic bacteria (B. succinogens) to filter paper cellulose indicating that simple phenolics may inhibit fibre degradation by interfering with the attachment of fibrolytic bacteria. Jung and Sahlu

(1986) also reported that when PCA and FA were esterified to cellulose at levels commonly found in forages, there was a reduction in IVDMD of the cellulose.

Simple phenolics also have been suggested to be toxic to bacteria, as the proportion of fermentation end products shifted when free vanillin or cinnamic acids were added to a mixed culture of rumen bacteria (Jung 1985; Varel and Jung 1986).

Jung and Fahey (1983) reported that free PCA and FA fed to rats at levels similar to those found in forages resulted in a negative correlation between food intake and level of phenolics. The effect of simple phenolics on reduced feed intake may not just be a problem with palatability. Glick (1981) indicated this with gallic acid. When gallic acid was infused into rats, it resulted in the same level of food intake reduction as seen when gallic acid is present in forages. Dietary gallic acid has been shown to be absorbed in the intestine of mammals (Booth et al. 1959). Several experiments by Martin (1982a,b,c) examined the effect of infusing simple phenolic acids into the abomasum of sheep, but feed intake was not measured in these experiments. It is important, given the high levels of phenolic acids in forages, that the effects of phenolics on palatability, feed intake and performance be studied in detail.

Lignin. One of the major factors affecting digestibility of forages is lignin. Akin (1982a) defined lignin as a polymer of phenylpropanoid units and states that it can

> "... vary in functional type depending on the predominate monomeric alcohol e.g. coniferyl or sinapyl used in forming the polymer."

There are two classes of lignin, core lignin and non-core lignin. Core lignin has two or more covalent linkages between phenolic monomer units within the lignin molecule (Jung 1989). Hartley (1972) described non-core lignin as lignin monomers which have one covalent linkage of the phenolic compound to either core lignin or hemicellulose and stated that non-core lignin is comprised mainly of FA and PCA. Both core and non-core lignin are linked to reduced digestibility (Jung 1989).

Drapula et al. (1947) reported that lignin appeared in ruminant feces, suggesting that the lignin was indigestible; thus, initiating the research into lignin and the effect of lignification on forage digestibility. Both lignin and cutin are resistant to anaerobic degradation due to their low oxygen content and tightly-packed structure (Van Soest 1982). These features also inhibit hydrolysis and aerobic catabolism. As well as being indigestible by rumen and intestinal organisms (Akin 1982a; Norton 1982), lignin can bind to fibre and reduce its digestibility by preventing the fibre from swelling and thus reducing microbe entry. Isolated lignin had no effect on microbial fermentation of forage; therefore, the reduced digestibility in the presence of lignin in forages was suggested to be caused by the complexing of lignin with CW components and rendering them unavailable to the microbes (Kamstra et al. 1958; Han et al. 1975). As well, exogenous application of lignin inhibited the digestion of cellulose and xylans by interfering with the attachment of bacteria to the cellulose and hemicellulose (Varel and Jung 1986; Theodorou et al. 1987). All functional types of lignin (based on the major component) increase in quantity as plants mature, but the rate of increase and/or order of increase is often different among tissues (Stafford 1962). The factors controlling lignification may

also vary in different tissues or even in the same tissue at various stages of development (Stafford 1962).

Using bromegrass, Casler (1986) found that lignin concentration was the greatest limitation to IVDMD, followed by cellulose and hemicellulose. Hacker (1982) also stated that CW digestibility was affected by the degree of lignification. Both CW content and lignin were found to have negative correlations with digestibility (Aman and Lindgren 1983). It is important to note, however, that lignin content per se is not solely the cause of lowered digestibility since legumes have higher lignin contents than grasses but are more digestible; therefore, the composition of lignin is just as (or more) important than the amount of lignin (Coleman 1983). Harbers et al. (1981) supported this statement by reporting that lignin in the mesophyll of leaves did not influence ruminal digestion. Earlier, Johnston and Waite (1965) had suggested that the site of lignification may be of greater importance than the quantity of lignin since orchardgrass had a higher lignin percentage than ryegrass at any digestibility and that a greater increase in lignin was required for a similar reduction in digestibility in orchardgrass.

Several interesting points concerning phenolic acids and lignin were raised in recent experiments with RCG. Burritt et al. (1984) reported high (0.89 to 0.91) correlations between lignin and PCA:FA ratio. Burritt et al. (1982) had earlier reported a correlation of 0.91 between lignin and PCA:FA ratio. Burritt et al. (1984) found that, over six harvest periods in one year, the PCA and the FA concentrations increased, as did the PCA:FA ratio, with increasing maturity. As indicated from the ratio, the PCA concentration increase was larger than the increase in FA concentration and the rise in

PCA was more consistent over the growth periods. Hartley (1972) had earlier reported that in ryegrass, the PCA concentration increased with maturity in a regular pattern, while the FA concentration increased for a time and then plateaued. Burritt et al. (1984) also found that the lignin concentrations did not conform to the expected pattern of a regular increase with maturity. The sixth harvest, which represents the milk stage, did have the highest lignin levels, but there were no constant increases between early leaf and heading stages. The data presented by Hartley (1972) and Burritt et al. (1982, 1984) suggests that higher PCA levels lead to increased lignification of the CW, since PCA exhibited a regular rise with age, as does lignin, in most cases. It had been reported that FA was bound to CW polysaccharides in lignified (Hartley 1973) and unlignified (Harris and Hartley 1976) cell types. Morrison (1974) stated that PCA served as crosslinking agent between structural CHO and lignin. Atsushi et al. (1984) and Azuma et al. (1985) indicated that PCA in forages was primarily esterified to core lignin and that FA was usually associated with hemicellulose via ether linkages. Scalbert et al. (1985) reported that PCA was associated with lignin usually through ester linkages and that FA bonded to hemicellulose with ester and ether linkages. From these findings, it appears that PCA is likely more involved in cross-linking of structural CHO and lignin than FA, due to the high correlation of the ratio of PCA:FA to lignification and the fact that PCA has a more regular increase with maturity than FA, as well as reports that PCA is linked to lignin. Ferulic acid does increase with age and therefore with lignin content, but since it plateaus, it has less effect. Kondo et al. (1990) also reported that FA had a small increase with maturity and they support Scalbert et al. (1985) who hypothesized that in primary

cell walls FA is ester linked to hemicellulose and that during lignification in the secondary CW, FA forms ether linkages with lignin.

Cell Wall/Lignin Interactions. It is impossible to discuss lignin and CW constituents separately since interactions between them will affect the digestibility. Isolated cellulose and hemicellulose are metabolized easily by rumen microorganisms or isolated enzymes (Dehority 1973; Smith et al. 1973; Williams and Withers 1982). Morrison (1979) found that polysaccharides in the CW matrix were rarely completely degraded and that the degradation varied with the tissue, plant species and plant maturity. Carbohydrate interactions with polyphenolics in the CW were reported to be the major inhibitors of CHO digestion (Van Soest 1981). Chesson (1983) hypothesized that the intrinsic nature of CW matrices affect CHO digestion to a greater extent than the physio-chemical characteristics of the CHO. Hatfield (1989) supported this concept by suggesting that bonding with lignin affected the extent of cellulose digestion and the degree of hydrogen bonding between the cellulose microfibrils affected the rate of cellulose digestion. As well, Hatfield (1989) added that there was likely to be a different interaction between the components in secondary CW formation than in primary CW formation. Morrison (1980) probably stated the case best when he suggested that the individual CW components should not be considered as separate units due to the bonding that exists among them.

Intake. Although this paper is focused on digestibility, it would not be complete without discussing how digestibility and the biochemical composition of the forage affect voluntary intake (VI). Blaxter et al. (1961) noted that low VI was associated with low

digestibility. The author also suggested that the reduced VI was inversely related to the increased travel time of the feed particles. Minson (1982) expanded on this idea by stating that the VI of forages by ruminants was affected negatively by bulk fill in the rumen, and that the bulk fill was related to the rate of passage of feed residues and the digestibility of the feed. Although there is a positive correlation between digestibility and VI over a wide range of forages, the correlation is not always as apparent from in vitro digestibility studies, thus reducing the accuracy of estimates of intake using these techniques (Hacker 1982). Crampton et al. (1960) reported that differences in VI accounted for 70% of the differences in the production potential of various forages and 20% attributed to the differences in digestibility of the forages, suggesting that factors other than digestibility affect VI.

Fibre is known to have negative effects on VI. Cell wall content is the most slowly digested portion and the least digestible of forage, and therefore, has the largest effect on rumen fill (Van Soest 1965). Van Soest (1965) reported that when the CW content of a forage was greater than 55-60% of DM, VI was reduced. When CW content was less than 50% of DM, the amount of CW had less effect on intake. These results supported the theory that there is a point where fibre does not significantly affect intake (Montgomery and Baumgardt 1965; Conrad et al. 1966) However, CWC is usually assumed to be inversely related to VI (Norton 1982). Negative correlations have been reported between VI and both ADF and NDF, but these correlations do not account for all differences in VI among forages (Minson 1982).

The effects of phenolics (PCA and FA) on digestibility has already been discussed. Since both FA and especially PCA have been linked to reduced digestibility, the presence of these phenolics in forages will lead to reduced digestibility, which will have some effect on reducing VI.

Lignin, as already mentioned, is totally indigestible by rumen microorganisms, thus the amount of lignin in forages will affect the digestibility on a dry matter basis. The interaction between lignin and CW components will affect the digestibility of the linked components and the overall digestibility of the forage and hence affect VI.

Anatomical Characteristics of Plants Affecting Digestibility

The anatomical type of plant material may affect the digestibility results. Stems, leaves, leaf sheaths and total herbage (mixture) usually have different digestibilities. Leaves are generally assumed to be of higher nutrient value and to have higher digestibilities (Norton 1982). After doing several studies, the author concluded that the concentration of CW on a DM basis is greater in stems than in leaves. For most species of forages there is less variation in leaf digestibility than stem digestibilities and heritabilities are lower (Hacker 1982). The author also suggested that since stem and leaf digestibilities are often positively correlated, some of the factors controlling digestibility should be the same for both leaf and stem. Wilman and Altimimi (1982), using Italian and perennial ryegrass, reported that leaf sheaths had digestibilities intermediate between leaves and stems.

Minson (1971) discovered that cultivars of Panicum spp. with higher leaf percentages provided greater voluntary intakes than those with lower leaf percentages even when the digestibilities were equal. The author suggested that differences in the physical properties, such as bulk density and surface area/g, of stems and leaves have greater effects on intake than the actual chemical composition. Laredo and Minson (1973) reported that the correlations between intake and leaves or stems were different at same levels of ADF, NDF and lignin. Leaf intake was significantly higher than stem in separated fractions even when the digestibilities were the same. Laredo and Minson (1975) theorized that the leaf fraction provided higher intakes since leaves have a faster rate of passage through the rumen than the stem fraction. As mentioned, the rate of passage affects bulk fill, which in turn affects voluntary intake. The authors also suggested that the faster rate of passage of the leaf fraction was due to the increased work required to break down stem versus leaf particles.

Increases in maturity are reported to result in a greater reduction in digestibility with stems than with leaves (Pritchard et al. 1963; Tilley and Terry 1964; Hanna et al. 1974). With maturity, there is a reduction in the proportion of young, digestible leaves, an increase in older, less digestible leaves and an increase in lignification, especially in the stem (Hacker 1982). Buxton and Russell (1988) reported that the lignin concentration on a CW basis doubled with maturity over that of immature stems in smooth bromegrass and orchardgrass.

Microstructure Components of Plants Affecting Digestibility

A review of the literature did not provide any information on the tissues of RCG and their digestibility. Thus, this section will refer to work with other grasses in order to provide a general introduction to the subject.

The major tissues in leaf cell walls of C3 grasses, also referred to as temperate or cool-season, are phloem (P), xylem (X), mesophyll (M), sclerenchyma (S), epidermis (E) and the vascular bundle (VB) defined as vascular tissue minus the P and X. Plants with the C4 system of photosynthesis are referred to as warm-season grasses and also have a well-defined parenchyma bundle sheath (PBS), which is also evaluated. Figure 1 and figure 2 indicate the above leaf tissues for cool- and warm-season grasses respectively. Akin and Burdick (1975) supported earlier research indicating that the P and M are rapidly degraded, E and PBS are slowly degraded and that the S, X and VB were non-degraded when using leaf sections digested in cattle rumen fluid and examined by scanning electron microscopy. However, there were differences in the rate of degradation among the same tissues of different species and cultivars; thus, the authors suggested that differences in the percent of the easily digestible tissues affected the rate of digestion. The rate of degradation would be affected by structural composition of the CW for the different tissues and different species. These differences in rate of digestion of the same tissues were also found in later research (Akin 1982a, Akin et al. 1983). For example, Festuca arundinaceae (tall fescue) cv. Kenhy has improved digestibility over an older cultivar (cv. Kentucky-31) and this improvement is related to an improved digestibility

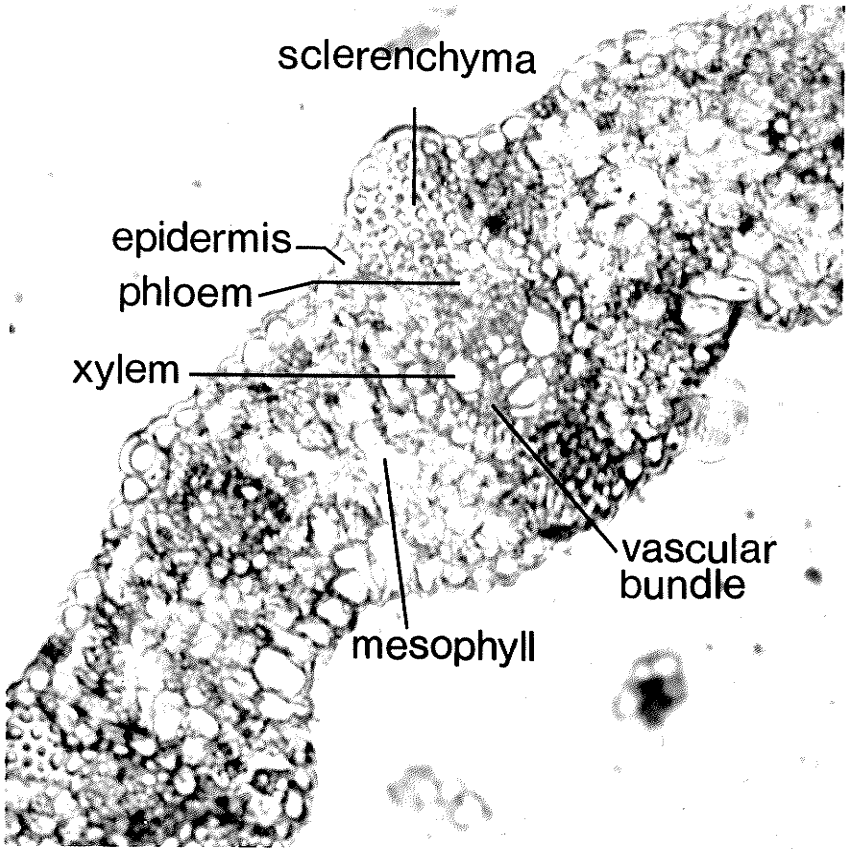


Figure 1. Timothy leaf cross section (22 microns) showing major tissue types in C3 grasses.

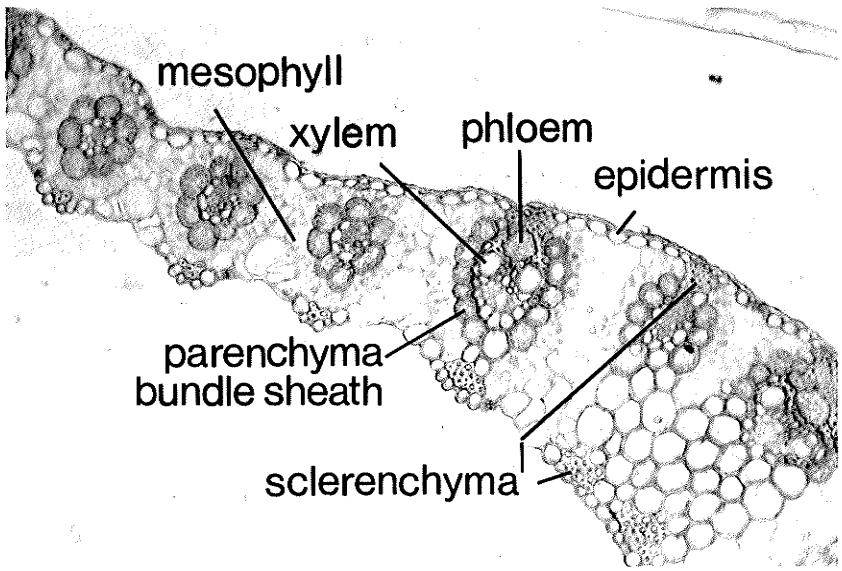


Figure 2. Switchgrass leaf cross section (22 microns) showing major tissue types in C4 grasses.

of the PBS (Akin 1982a). Akin (1979) also reported differences among species and cultivars in rate of digestion of the same tissues and suggested that there are genetic differences in CW composition that affect digestibility. Harbers et al. (1981) also reported that the presence of lignin in smooth brome and tall fescue grasses resulted in more time required for digestion of S and E and prevented digestion of the X and VB but had little effect on the digestion of readily degraded tissues. The authors suggested that both the amount and location of lignin are important in forage quality evaluation.

In stems, the major cell wall tissues are the sclerenchyma ring (SR), parenchyma (PA), cortex (C), E, VB, X and P. Figure 3 (page 53) shows a RCG stem cross section with the above tissues indicated. After incubation in rumen fluid for 48 hours, the E, SR, X and VB were left undigested (Akin 1989). The digestibility of the PA varied with maturity. In young stems it was rapidly degraded while in older stems it was slowly or partially degraded. Akin and Burdick (1981) had previously reported that the E, SR and VB were undigested. Schank et al. (1973) reported a significant negative correlation between IVDMD and the percent of vascular tissue in stems. The authors also reported that an increase in maturity resulted in increased lignification of the vascular bundle but there was no increase in the size or area. Most research has focused on leaf digestibility, hence there is little information on stems.

Histochemistry is one of the quickest methods of determining the presence of lignin. Given the relationship between lignin and digestibility, histochemistry may be used as a tool to predict digestibility. The two most common stains used in the literature are acid phloroglucinol (AP) and chlorine sulphite (CS). Upon reacting with the coniferaldehyde

groups of lignin, AP turns a deep red to purple colour, but it gives a poor reaction with syringyl units of lignin (Stafford 1962). Chlorine sulphite gives a red colour when it reacts with syringyl units (Stafford 1962). In an experiment with Panicum spp., Akin and Burdick (1981) reported that the undegraded tissues in stems (E, SR, X, and VB) gave positive reactions with AP and, as mentioned, were undigested. The PA gave variable reactions reflecting its variable digestibility. In the lower stems of Panicum spp., Akin et al. (1984) reported some positive reactions with CS for PA and also reported varying digestibilities for the tissue. Akin (1989) suggested that these tissues are the most consistent and active barriers to digestion and are likely resistant to microbial colonization as well as degradation. The cell walls of some leaf tissues, e.g. PBS of bermudagrass, PA, abaxial E and occasionally the M of tall fescue, were slowly or partially degraded and stained positive with CS but not AP (Akin and Burdick 1981). The authors also reported that leaf tissues that produced positive reactions with AP were undigested (VB, X). Akin (1989) reported similar results where there was partial degradation of leaf S which stained CS positive and no digestion of E and SR (stems) and VB (stems and leaves), all of which stained positive with AP. These results suggest that syringyl units have a different reaction with cell wall constituents than coniferaldehyde units and both lignin units may influence digestibility.

A recent study by Akin et al. (1990) utilized several diazonium compounds as well as AP and CS stains. The authors reported that, in general, the diazonium stains gave tissue reactions similar to CS but not AP. The diazonium stains were also recorded as being more effective than CS and AP in providing colour reactions with phenolic acids,

phenolic aldehydes and phenolic esters.

The number of VB have also been reported to be related to digestibility. Schank et al. (1973) indicated that in Hemarthria altissima lower digestibilities were correlated with a larger proportion of VB in the stem. The number and the frequency of VB were reported by Tan et al. (1976) to exhibit genetic variation in Bromus inermis, but the relationship between the VB and digestibility was not examined. However, Akin and Burdick (1973) and Hanna et al. (1974) reported that bermudagrass genotypes which had significantly different digestibilities did not differ in microstructure in either leaves or stems.

Methodologies for Determination of Forage Quality

Coleman (1983) defines three main requirements for techniques used to evaluate plant quality. Firstly, the techniques need to be reliable in their prediction of plant value. Techniques also need to measure characteristics relevant to current farming practices. To be useful to plant breeders who evaluate hundreds of different plants, the techniques should also be relatively simple and produce quick results.

In Vivo Techniques

In vivo and in situ techniques provide the only truly accurate estimations for forage digestion; although there still are small inaccuracies due to loss of very small particles

through the nylon bags. These methods are very expensive due to the number of animals and the time required to evaluate sufficient samples for plant breeding or plant selection research. In vivo systems also require large sample sizes which are not always possible to obtain due to space limitations for growth of plant material. Thus, laboratory techniques for evaluating digestibility are more commonly used.

In Vitro Techniques

In vitro digestion of plants with rumen microorganisms or enzymes are also used to obtain values for the digestibility of forages. These results are usually highly correlated with in vivo results.

There are many variations of the in vitro digestion technique. The common goal is to attempt to simulate what happens to the forage in the rumen. In vitro systems are considered to provide a more accurate estimation of digestibility than chemical methods, since these systems, allow for, as yet, unknown factors within the rumen that influence both the rate and extent of digestion (Van Soest 1982). The two-stage in vitro technique (Tilley and Terry 1963) had greater accuracy than previously used chemical techniques (Coleman 1983). Cellulase digestibility studies have shown good correlations with in vivo results (Jarrige et al. 1970; Jones and Hayward 1973; McLeod and Minson 1978; Bughara and Sleper 1986; Casler and Sleper 1991). However, in vitro systems tend to be more expensive and require a longer time to conclude than most chemical methods. For example, incubations using either rumen fluid or specific enzymes are often forty-

eight hours in length. As well, most in vitro methods have poor reproducibility due to inconsistencies within the technique despite attempts to eliminate these inconsistencies, such as the varying activity of rumen fluid between and within runs, even from the same animal fed the same diet (Tilley and Terry 1963). Minson (1987) suggested running samples with known in vivo digestibility and adjusting the values obtained from the in vitro analyses to match the in vivo. This would provide some standardization between runs.

All in vitro systems use some form of digestion end products or dry matter loss for the calculations of digestibility. Gravimetric systems use residual dry matter, while other systems use volatile fatty acid or gas production. Van Soest (1982) provides an excellent review of all major systems currently in use, and summarizes by stating that not all systems are of equal value and that the selection of a system should depend on the future use of the results. Again, the only accurate way to determine digestibility is to feed the plant to the animals, but as mentioned, in vivo results are expensive and time-consuming to obtain.

Techniques for Biochemical Analysis

Chemical methods are often used to examine levels of digestible, semi-digestible and indigestible components of plants and to correlate these results to in vivo digestibility results. Some chemical methods, such as histochemistry or lignin values, also may be used to predict digestibility. This section will outline the current methods in each

category and discuss some of the main advantages and disadvantages of each method.

One of the earliest chemical quality techniques is the proximate analysis system which provides a large amount of nutritional information; however, this system tends to produce numerous and often high errors (Van Soest 1982). The detergent system of analysis (ADF, NDF) (Van Soest 1963) was designed to replace proximate analysis but some precision and detail were lost (Van Soest 1982). Surprenant et al. (1988) also stated that the detergent system was slow and expensive; thus, it is not useful for analyzing large numbers of samples.

Lignin analysis tends to be more difficult in theory since lignin is not a uniform component as it varies in chemical make-up and complexity. No single analysis has been found to be totally satisfactory (Monties 1989). The limitations of the various methods have already been outlined (Browning 1952; Browning 1957; Lai and Sarkanen 1971); however, the major points will be reviewed here. The Klason lignin procedure, which is basically the precipitation of lignin after sulphuric acid treatment, is often contaminated with protein and sugar moieties. There are also various spectroscopic methods, such as ultra violet detection, but these are limited by the accuracy in determining the absorbance coefficient (Monties 1989). The detergent fibre methods, which are variations of the Klason method, employ various pretreatments to remove lipids, proteins, xylans and phenolics other than lignin (Van Soest 1963). There are, however, inaccuracies involved with the extraction conditions and the time and temperature of the reactions (Monties 1989). Another method of lignin determination is the permanganate lignin procedure. Goering and Van Soest (1970) state that the permanganate method takes less time to

obtain lignin values than the detergent method, and the residue is reserved for the analysis of cellulose and silica, and that the reagents are less corrosive and do not require standardization as do the detergent reagents. There is also less interference from the use of the 72% sulphuric acid. The authors also state that there are no filter aids required for the permanganate method and the results are less affected by heat damage artifacts. Due to these differences between the permanganate and detergent methods, Goering and Van Soest (1970) state that the permanganate method tends to produce lignin values that are closer to a "true" lignin value.

As mentioned earlier, the usefulness of any method depends upon consistent statistical correlations between the analyzed components and rumen digestibility. Prediction equations using regression techniques can also be employed by applying previously obtained values to predict digestibility. A major disadvantage of using chemical methods for digestibility predictions is the inconsistent relationship between nutritional components and digestibility between experiments (Van Soest 1982). No one component has been shown to have a consistent relationship with digestibility over a large number of experiments. There is always some relationship, but the actual values of the correlations vary, and with small sample numbers, the inconsistency is increased. The correlation values between biochemical components and in vivo digestibility are usually different for the different forage species; there is no one regression equation that works over all forage species (Burritt et al. 1984).

While the use of near-infrared (NIR) analysis of forage samples is not "new", it is only recently that NIR has received significant interest by plant breeders. The first report of

using NIR for the prediction of forage quality was published by Norris et al. (1976). The basic principle of NIR involves using a computer and an optical device to record the reflectance spectrum of feed samples with known composition. This data is then used to produce equations which can be used to predict the composition of unknown feed samples based on their reflectance spectrum. While most proponents of NIR maintain that it is precise, accurate, rapid, simple and cheap, not all researchers agree. Firstly, excellent calibration with standards of similar composition is required or else very large errors may result (Van Soest 1982). It is true that once the laborious, time-consuming task of obtaining accurate results from wet chemistry to establish the composition of standards, and after determining the optimum prediction equations, the analysis of the unknown samples is indeed rapid and simple (Flinn and Murray 1987). Large numbers of samples with similar composition are required in order to justify the expense of the equipment (Van Soest 1982). A definite advantage of NIR is indicated when only small amounts of sample are available, in that NIR is non-destructive, thus leaving the sample available for further analyses (Flinn and Murray 1987). Because NIR only provides information once the appropriate wavelength and/or prediction equations are determined, it is not a diagnostic tool and is not useful for research involved with determining how a specific component affects forage digestibility (Van Soest 1982). However, the use of discriminate analysis with NIR will allow researchers to search for particular substances in feed samples once the optimum wavelength is determined. Near infra-red analysis is thus best for plant evaluation programs where large numbers of similar samples are investigated for basic nutritional composition (ADF, CWC, NDF).

Techniques for Microstructure Analysis

In the research field, it has been recognized that the major problem with relating composition of feeds to feeding value is that plant CW structure as well as the concentrations of nutritive components affect digestibility. The digestion of the CW depends not only on composition of tissues but also on the character of the components, that is the presence of barriers to microbial attachment and degradation (Van Soest 1982). To examine these barriers, both physical and chemical, various microscope techniques and types have been developed. Akin (1979) provides a review of microscope types and their use in plant quality research. Use of the light microscope

> "...reveals the plant tissue arrangement, amounts of tissue types, cell wall sizes, and, with specific stains, the sites and types of lignified or non-degradable tissues." (Akin 1979).

The scanning electron microscope (SEM), which provides a surface analysis of plant sections, can be used to provide information on rate and extent of digestion of various tissues over time. Modes of association of rumen microbes with plant cell walls and the digestion provided by each type of microorganism (protozoa, bacteria, fungi) can be examined by transmission electron microscopes. The expense of the equipment and the complexity of sample preparation need to be considered before implementing any of the above (Akin 1979).

Histochemistry, like NIR, has been used for many years but is still being improved. Currently, histochemistry has little usefulness to researchers except as a ranking tool

since most stains are not specific to one compound and the stains do not accurately quantify the amount of lignin. For example, there is not a stain that will define ferulic acid only.

Akin (1982c) published a section-to-slide (STS) technique for the digestion of thin sections placed on standard microscope slides and determining the extent of tissue digestion over time or at one particular time. This method is less time-consuming than SEM and gives sufficient information for most experiments. In conjunction with image analysis, STS procedures are increasing in use.

The use of image analysis (IA) procedures in forage research is increasing. Image analysis allows for calculated determinations of tissue area rather than relying upon rough estimates by eye. Cell wall area can also be precisely calculated and this data may be used to provide more information about cell wall degradation (Twidwell et al. 1989). Regarding digestion studies, the use of IA has significantly improved the accuracy and reduced the time necessary to perform these studies. Before IA, the extent of digestion of plant sections was estimated by eye using 2 or 3 people to obtain an average. Now with IA, accurate values can be obtained by only one person and requiring only about 15 minutes per slide (Twidwell et al 1989).

Image analysis has also improved lignin determinations. The quantity of lignified tissue can now be determined using a lignin stain with IA instead of just reporting where lignin is located (Kuhbauch and Bestajovsky 1985; Twidwell et al. 1989). Kuhbauch and Bestajovsky (1985) reported high correlations (0.90 to 0.96) between IA results and lignin concentrations using the method of Goering and Van Soest (1970) and in vitro

digestibility by Menke et al. (1979) (0.84 to 0.92). These results suggest that IA may be able to replace wet chemistry results for lignin and in vitro digestibility determinations once the correlations are proved to exist for other species of plants. One possible problem with IA for lignin determinations, especially with grasses, is that if the lignin is not located in one specific area or in a number of large areas, it can be time-consuming to trace all the lignified areas (Kuhbauch and Bestajovsky 1985). With the true-colour discrimination, however, the user can pick the colour to be examined, or use all shades of a stain, and the process then takes only several seconds per section. In an experiment by Wright (1989) to examine bone marrow fat content using both IA and subjective analysis, they reported high correlations between the methods. Overall, the use of IA depends on whether the number of samples, existence and availability of equipment, available personnel and the degree of accuracy required justify the expense of initiating IA. In plant research or plant breeding, the number of samples and the need for accuracy do justify the expense.

Potential for Improvement with Genetics

Selection Criteria

Burton (1970) stated that, when selecting plants for breeding programs, scientists must consider yield; nutritional composition; presence of toxic compounds, such as alkaloids and tannins; disease and pest resistance; environmental adaptation; ease of establishment;

adaptation to different management systems; and production of propagation material. While most breeders select on one of the above, before endorsing the improved plant material, the other characteristics should also be considered.

As mentioned earlier, it is essential in programs where many plants are evaluated, that the screening techniques be rapid, routine, use small sample sizes, provide a precise prediction of the character in interest, and be reasonably low cost (Coleman 1983). In order to have improvements in forages, breeders need genetic variability among cultivars and among genotypes for the selection criteria (Casler 1990). Cooper et al. (1962) suggested that using species with lower digestibilities may provide a greater margin of improvement than those with higher digestibilities. Casler (1987) stated that nutritional improvements of forages, especially in energy, will require changes in the digestibilities or relative concentrations of CW constituents. Many species of forages show greater differences in digestibility at more mature stages of growth than at earlier stages (Schank et al. 1973; Klock et al. 1975; Sleper and Mott 1976); however, a few species have been reported to have similar digestibilities in young and more mature forage (Hacker and Minson 1972). The research presented below will show the potential for improvement through genetics, focusing mainly on RCG and with some examples from other grasses to complete the picture.

Reed Canarygrass Breeding

Reed canarygrass is not currently a noted agriculturally-important grass due to fairly low palatability and low voluntary intake (Marum et al. 1979). Breeding to reduce alkaloid content in RCG and to improve animal performance has put digestibility and intake as the major limitations to RCG (Surprenant et al. 1988). Digestibility has been shown to be a highly heritable trait in RCG (Carlson et al. 1969; Williams et al. 1971). Intake can be improved by increasing the stocking rate in RCG fields, since animals tend to prefer other grasses to RCG but will graze RCG uniformly if not given a choice (Miller 1984). Thus it is likely that with further research, RCG will become a useful and important forage.

Reed canarygrass is considered by most geneticists to be a diploid which makes it an easy plant to work with (Marum et al. 1979). Numerous studies have been performed recently to examine the variation in both agronomic characteristics and quality factors in RCG.

When breeding for quality, it is important to remember that not only must the plants be of sufficient quality, but that they must also be economical to produce. Thus some recent research on variation in agronomic characteristics and the possible effects of breeding for improved digestibility will be presented. Hovin et al. (1976) reported highly significant differences among parental genotypes of RCG for yield of forage, but no differences were observed in the progeny. Sachs and Coulman (1983) determined that there was significant variability in heading date and height in populations of RCG in

eastern Canada, but no variation in fall or spring vigour or in yield across cultivars and cuts. Seven populations of RCG were reported to differ significantly for all forage quality traits (yield/plant, yield/area (g/m), ADF, NDF and CP in the vegetative state (Surprenant et al. 1988). In RCG plants selected for high specific leaf weight or low specific leaf weight, total herbage production was not significantly altered over various harvest dates or years (1 or 2) (Buxton 1990).

Hovin et al. (1976) reported low correlations between both IVDMD and CWC and yield, suggesting that no significant reductions in yield would result from selecting for these characteristics. Marum et al. (1979) also reported small negative correlations between both IVDMD and CWC and yield and, therefore, concluded that selection for higher IVDMD or lower CWC would not have significant effects on yield.

Among parental genotypes of RCG, Hovin et al. (1976) found highly significant differences for CWC and significant differences for IVDMD. When the crosses were examined, the authors found highly significant differences for both CWC and IVDMD. Marum et al. (1979) concluded that the presence of additive genetic variation should provide for improvements in selection for CWC, IVDMD and yield. When parent genotypes were ranked for CWC and IVDMD, Hovin et al. (1976) consistently found that low CWC plants had high IVDMD (often with slightly lower yield), thus substantiating the use of parent genotypes in evaluation programs for RCG. Given the above research results, it is unlikely that breeding for improved digestibility will significantly affect yield.

Marum et al. (1979) reported high positive correlations (0.88) between CWC and both

lignin and ADF. Negative correlations (-0.81 to -0.91) were reported for CWC and both IVDMD and CP content. Marum et al. (1979) provided correlations for IVDMD with ADF (-0.92), lignin (-0.88) and CWC (-0.85) and suggested that since the correlation was highest for ADF, ADF is the best chemical method to predict IVDMD. This agrees with previous research (Hovin et al 1976).

A very high correlation (0.97) was found between CWC and ADF which suggests that selection for lower CWC should result in a similar reduction of ADF, and that selection for either ADF or CWC should give approximately the same increase in IVDMD (Marum et al. 1979). Ideally a reduction in CWC should lead to an increase in cell contents (soluble CHO, lipids, protein etc.) which are predominantly readily soluble, thus, increasing digestibility which may lead to an improvement in intake (Hovin et al. 1976, Marum et al. 1979). Christensen et al. (1984) reported large positive correlations between total digestibility and both leaf and stem digestibilities, suggesting a strong relationship between all three characters in RCG. Thus, the authors state that there is no benefit to selecting for higher stem IVDMD than for total IVDMD in RCG. Ostrem (1988) reported high broad sense heritabilities (0.60) for IVDMD over all genotypes and all populations of RCG tested, which suggests that higher IVDMD values should result from selection of plants with higher IVDMD.

In several populations of RCG, Surprenant et al. (1988) found significant variation for NDF, ADF and CP content. Selection for RCG genotypes with low NDF resulted in improved values for ADF and CP content (Surprenant et al. 1988). The authors also reported that selection for low NDF significantly reduced the density of growth; therefore

a reduction in yield may occur in RCG plants selected for lower NDF. However, the research above suggests that only small reductions in yield will occur when selecting for higher nutrient value.

Thus, given the substantial variation in both agronomic and nutritional characteristics in RCG, and strong correlations between nutritional characteristics, there is no doubt that RCG can be further improved with breeding research.

Breeding for Quality in Other Forages

Because warm season grasses are generally thought to have lower digestibilities than cool season grasses, most research has focused on improving the digestibilities of these grasses. Burton and Monson (1981) reported an increase of 34% in digestibility with Coastcross-1 bermudagrass over coastal bermudagrass using the nylon bag technique. Other warm season grasses have been improved using in vitro digestibility techniques. An example is pearl millet (Burton and Monson 1981). Vogel et al. (1981) reported that a newly developed genotype of switchgrass had a higher IVDMD than an unimproved genotype. Panicum spp. with C3 photosynthesis provided highly significant negative correlations between both the percentages of PBS and vascular tissue (VT=VB-phloem) and IVDMD (-0.53 to -0.88) (Wilson et al. 1983). The authors also reported high positive correlations (0.62 to 0.63) between M content and IVDMD. These results suggest that breeding for reduced PBS and VT and/or increased M should result in increases in the IVDMD.

The digestibilities of several cool season grasses were shown to be under genetic control (Ross et al. 1970; Cooper 1973; Coulman and Knowles 1974; Cooper and Breese 1980; Hoveland and Monson 1980; Collins and Drolson 1982). Casler (1987) reported that in smooth bromegrass the in vitro NDF digestibility showed strong relationships with the in vitro cellulose and hemicellulose digestibilities; therefore, the author suggested that direct selection for in vitro NDF digestibilities should also increase cellulose and hemicellulose digestibilities. Plants with increased cellulose and hemicellulose digestibilities may have reduced lignin content, and this should result in greater availability of cellulose and hemicellulose to rumen microbes. After breeding for increased IVDMD, Casler and Carpenter (1989) reported significant differences of 36-42 g/kg DM between high and low IVDMD strains of smooth bromegrass, using only stem material. Leaf samples resulted in differences of only 13-17 g/kg DM, again suggesting that changes in IVDMD of total herbage is more affected by changes in stem digestibilities than leaf digestibilities. The authors also reported that the increase in IVDMD in stems was associated with a reduction in the NDF content, thus supporting the hypothesis of Casler (1987). Thus, there is definite potential for using plant breeding to improve forage digestibility.

MATERIALS AND METHODS

GROWTH EXPERIMENT

The plant material used in this study consisted of the nine parental genotypes of the RCG (Phalaris arundinacea L.) cultivar Rival which had been selected as being free of the tryptamine alkaloids and relatively low in total alkaloid content (Agriculture Canada Food Production and Inspection Branch License number 2572). Material was provided from stock plants held at the University of Manitoba. One pot of each was transported by car to the Central Experimental Farm in Ottawa. The plants were broken into tillers and potted in 5" plastic pots with one tiller per pot. All tillers were at approximately the same stage of growth. Plants were moved to a growth cabinet and arranged in a Randomized Complete Block Design (RCBD) with four replicates. The cabinet was set at 25°C for a 16 hour day and 20°C for the dark period. Lighting was provided by a mixture of incandescent and florescent bulbs. After four weeks of growth, the number of fully expanded leaves was recorded every other day for three tillers per pot and the total number of tillers in a pot was counted once per week until material was harvested for the gravimetric procedure.

Growth characteristics were analyzed as a RCBD with the Statistical Analysis System (SAS) (1985) by the Analysis of Variance (ANOVA) procedure. The Student-Newman-Keuls test was used to detect differences among genotype means at $P \leq 0.05$.

Gravimetric Digestibility Analysis

The plant material used in this experiment was the same material grown for the growth experiment. Plant material was harvested when three stems per pot over all replicates of one genotype reached six fully expanded leaves, which equates to stage 16 of Simon and Park (1981). The individual plants were harvested above the first node. Stems plus sheath fractions were cut into 1 cm segments for the digestibility study (10 segments per plant). Leaf material, used in this study, consisted of the second oldest fully expanded leaf. Again, ten 1 cm pieces per leaf were collected. The fresh 1 cm leaf and stem pieces were placed in 2 labelled, pre-weighed 60 ml Vulcan centrifuge tubes, one for leaves and one for stem material. Tubes were capped tightly and dipped in liquid nitrogen until thoroughly frozen. The frozen leaf and stem fractions were stored at -80°C for at least 24 hours prior to freeze-drying.

The frozen tubes and plant material were freeze-dried for 48 hours in an upright freeze drier (Virtis, model 10-145 MR-Ba, Gardiner, New York) with the caps removed and a double-folded piece of cheesecloth tightly wrapped over the tubes to prevent loss of sections. Tubes with contents were weighed, after which plant material was evaluated for digestion using a 2:1 mixture of McDougall's buffer and rumen fluid.

Buffer was prepared according to McDougall (1948) and stored at 39°C until combined with rumen fluid. Rumen fluid was collected from a cannulated sheep fed a diet of mixed grass hay and haylage. The complete diet is recorded in Appendix 1. Rumen fluid was blended for 2 minutes in a standard food blender, strained through 4

layers of cheesecloth and filtered through glass fibre. All procedures were performed with the equipment and rumen fluid held at 39°C and in the presence of carbon dioxide. The rumen fluid was then examined under a light microscope for assessment of quantity and viability of protozoa. The presence of live protozoa is an indication of active rumen fluid, since protozoa are considered to be the most sensitive to pH and temperature changes (Ames, personal communication). Twenty ml of rumen fluid and 40 ml of buffer were dispensed into each tube, gently mixed, capped and placed into a controlled environment incubator/shaker (Model G25, New Brunswick Scientific Co., Edison, New Jersey). Incubator temperature was maintained at 39°C. Leaf samples were incubated for 24 hours and stem samples for 30 hours. These times were chosen based on the results of a preliminary gravimetric digestibility experiment designed to indicate which times of incubation resulted in near maximum digestion.

Following incubation, tubes were removed from the incubator and placed in cold water to stop microbial activity. Rumen fluid was strained off through cheesecloth and the remaining plant material was rinsed with distilled water three times. Tubes and remaining plant material were frozen at -80°C for at least 24 hours prior to freeze-drying (48 hours), after which tubes were placed in a desiccator until weighed. The percent dry matter loss due to digestion was calculated by the following equation:

%DM = ((initial freeze-dried weight - digested freeze-dried weight)/initial freeze-dried weight)*100

The results were analyzed using the ANOVA procedure of SAS and the Student-Newman-Keuls test was used to identify mean differences among genotype digestibilities at $P \leq 0.05$.

ALTERNATIVE TECHNIQUES STUDY

Experimental Design and Set-up

Based on the results of the gravimetric digestibility study, two low digestibility genotypes, designated as 91 and 80 and two high digestibility genotypes, designated as 83 and 96 were selected for further evaluation.

The four genotypes were evaluated at three stages of growth, namely the four, six and eight fully expanded leaf stage which correspond to stages 14, 16 and 18 denoted by Simon and Parks (1981), which will be denoted as growth stages 1, 2 and 3 respectively. For each genotype, six plants were individually transplanted into 1 litre milk cartons containing Turface (Aimcor, Deerfield, Illinois), an inert growth medium. These modifications from the previous growth experiment were employed to ensure that sufficient root growth occurred to maintain the plants to the eight leaf stage (growth stage 3) and to reduce the risk of disease. Four drainage holes were drilled into the cartons. The tillers were at approximately the same stage to reduce individual variation. The material was placed in a controlled environment growth chamber set to receive 16 hours per day of 330 micromoles/m2/s light from a mixture of incandescent and cool

fluorescent lamps with a temperature regime of 23/18°C day/night. Lights were set at their highest point to encourage stem elongation. Plants were fertilized with 20-20-20 twice a week. One week after planting, dead tillers were replaced with live tillers where material was available.

The experiment was planned as a RCBD, arranged as a split-plot, replicated four times. Genotypes were the main plots and growth stages were the sub plots. Due to space limitations, replicates 1 and 2 were grown before replicates 3 and 4, the latter of which was used for all laboratory analysis procedures, except for the lignin/ADF procedure where all four replicates were analyzed. The growth cabinet and all settings were maintained throughout the experiment. The genotype by replicate (geno*rep) interaction was used as the error term for the genotype effect, and the growth stage by replicate within genotype (growth stage*rep(geno)) interaction was used as the error term for effect of growth stage and growth stage by genotype (growth stage*geno) interaction. The individual plants were used as the experimental units.

Sample Harvest and Preparation

Plants were harvested when a minimum of 4 of 6 plants across all genotypes and replicates had achieved the required leaf numbers for each stage of growth. Plants were harvested above the first node. Five 2.0 mm pieces were cut from the stem above the original cut, placed into labelled glass vials, immersed in liquid nitrogen and placed on dry ice until being moved to the - 80°C freezer. The leaf sheath was removed from the

stem pieces. Stem material collected from each plant was kept separate. Leaves were stripped from the remaining stem material, leaving the leaf sheath intact on the stem, and cut into smaller pieces and put in labelled paper bags in preparation for lignin analysis.

Lignin and ADF Analysis

The stem plus sheath fractions from the six plants within each cut and replicate were combined to ensure adequate sample for the lignin procedure. The leaves were discarded. Sample material was dried at 100°C for one hour and then at 60°C for another 47 hours. Dried material was ground to pass a 40 mesh (40 holes per inch) in a countertop model grinder (General Electric) and stored for lignin analysis.

The ADF and acid detergent lignin content of the sample material was determined using the method of Goering and Van Soest (1970). The only modification was that glass fibre was not used, as the error between runs was lower without including glass fibre.

Section Preparation

One of the 2 mm pieces of stem was embedded in Tissue-Tek (O.C.T Compound, Miles Canada 4583) and sectioned at a thickness of 16 microns using a Microtome-Cryostat (Minotome Model 115, Damon/International Equipment Company, Needham Heights, Massachutes). Tissue-Tek is described as a formulation of water soluble glycols and resins and does not interfere with microbial activity or staining.

Approximately six to eigth 16 micron sections were placed on glass microscope slides for evaluation of specific tissues by histochemical reactions for lignin. One slide was used for acid phloroglucinol (AP) and one slide for the chlorine sulphite (CS) stain. For evaluation of tissue digestibility, approximately 6 to 8 16 micron sections were placed on slides which had a strip of double-sided tape attached. All three slides were prepared from the same stem piece. Finished slides were stored at -20°C until used.

Histochemical Analysis

The AP staining preparation was based on that described by Jensen (1962). The AP solution was modified from the 1% solution used by Jensen to a 2% w/v solution (acid phloroglucinol powder in 95% ethanol) which was thoroughly mixed with 10 M hydrochloric acid in a 1:2 AP solution to acid ratio. The AP solution was prepared fresh every three hours to maintain full efficacy.

The slides for the AP procedure were removed as needed from the freezer and given several minutes to defrost. Enough AP stain was put on each slide to immerse all the sections. The stain was left on slides for 3 minutes and drained off. Sections were covered in spectra-analyzed glycerol to enhance colouration and to keep the sections from drying out, thus maintaining them for the image analysis procedure. The slides were covered with glass coverslips. Excess glycerol was wiped off with a tissue and the slides were sealed with clear nailpolish.

Tissue reactions were evaluated visually under a light microscope with a 10 power objective lens. The ranking system for colour reactions was similar to the 0 to 3 system used by Akin et al. (1986), except that this scale went from 0 to 7 to allow for lighter or darker shades of a colour. For most of the colours, the Red/Pink colour chips developed by Kelly (1965) were used to ensure standard colour scores. The complete set of colour chips and the information concerning them can be found in Turnbull (1980). All sections on a slide were examined and one score was assigned for each tissue on each slide.

Tissues examined were the epidermis, cortex, sclerenchyma ring, phloem, xylem bridge (defined as the transversing band of cells bisecting the phloem and xylem vessels) (Akin and Burdick 1973), vascular bundles (vascular tissue minus the phloem and xylem bridge and xylem vessels) and the parenchyma. Figure 3 shows a RCG stem section labelled with all tissues described above, except for the xylem bridge. Visual observation of stained sections was done within 5 minutes of sample preparation to prevent errors associated with fading of the colour with time.

The CS stain was also modified from the procedure described by Jensen (1962) by changing the sodium sulphite solution from a 1% w/v solution to a 2% w/v. The sodium sulphite solution was prepared and refrigerated at 4°C for at least 30 minutes, since it needs to be cold to work efficiently. A small amount of Javex brand commercial bleach was acidified to pH 1 with 10 M hydrochloric acid. Both solutions were freshly prepared after three hours to maintain full effectiveness.

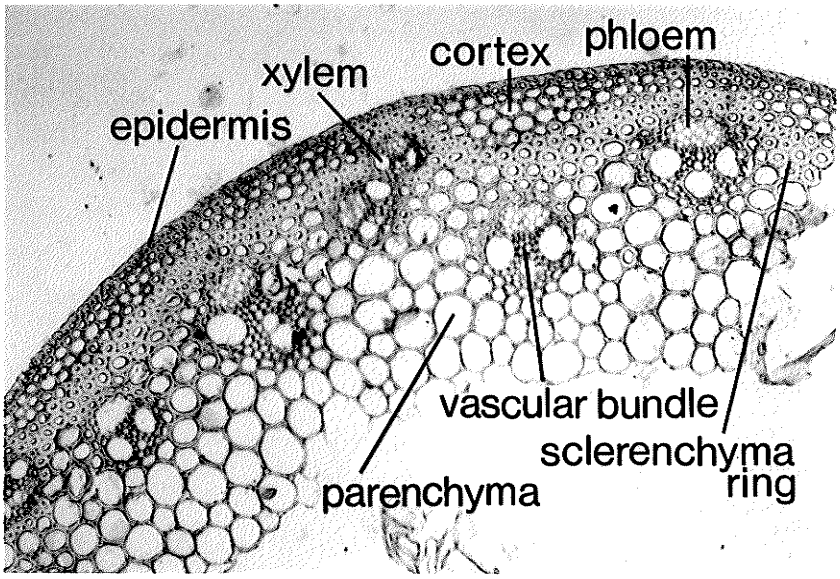


Figure 3. Reed canarygrass stem cross section (22 microns) showing major tissue types in C3 grasses.

The slides for the CS work were taken out of the freezer as needed and allowed to defrost for several minutes. Enough of the bleach solution was put on each slide to cover the sections. This solution was left on the sections for 10 minutes. After 5 minutes, slides were checked to ensure that sections were still immersed. Following the 10 minute immersion, the bleach solution was rinsed off using distilled water, and sections and slides were immersed in distilled water. The slides were then carefully dried and the cold sodium sulphite solution was added to each slide in quantities sufficient to immerse each section. The sodium sulphite solution was left on the slides for 3 minutes. At the end of 3 minutes, the solution was carefully wiped off, and several drops of spectra-analyzed glycerol were put on each slide. Slides were covered with coverslips and sealed with clear nailpolish.

Tissue reactions were evaluated with a light microscope using a 10 power objective lens. The same tissues were examined as in the AP procedure. Since none of the colour chips matched these colours, a paint chip from Home Hardware was found which provided samples of most of the colours. Again the scores ran from 0 to 7. All sections on each slide were examined to remove any discrepancies from section to section, and each tissue for the slide was given one score. Slides were scored within 5 minutes after preparation to minimize error associated with fading of colour.

Section-to-Slide

This technique was based on the one described by Akin 1982c. Rumen fluid was collected from a fistulated cow fed a diet of hay and grain (Appendix 1). The fluid was strained through 4 layers of cheesecloth and a small amount was viewed under a light microscope for the presence of active protozoa to ensure microbial activity. Fifty ml of rumen fluid were added to glass Coplin jars (Fisher, Catalogue number 08-815) containing the previously prepared slides for incubation. Plants from each genotype by growth stage combination were kept together in one jar to allow identification if the tape fell off the slide. All procedures were carried out in the presence of carbon dioxide. Jars and slides had been incubated at 39°C, prior to the start of rumen fluid collection, to reduce temperature shock. Each jar was breezed with carbon dioxide and sealed with a number 10 rubber stopper equipped with a one-way valve to allow gases produced during incubation to escape. A piece of parafilm (American Can Co., Greenwich, CT) was wrapped around each jar to ensure a tight seal around the stopper. Several slides with 16 micron sections of timothy leaves were also incubated as standards. The jars were placed in an upright incubator preheated to 39°C.

The jars were removed from the incubator after 24 hours of incubation. Timothy leaves were examined first for tissue loss. Previous experiments (Ames, personal communication) had shown that the majority of leaf tissues are digested within 24 hours. On these particular slides only the chlorophyll pigments and the vascular bundles (minus the phloem) were left undigested. Since the timothy leaves had been appropriately

digested, it was assumed that the procedure had been performed accurately and that digestion had taken place. The digested RCG slides were removed, rinsed with distilled water, and excess water was carefully removed with a tissue. Several drops of spectra-analyzed glycerol were added to each slide, the slides were covered with glass coverslips and sealed with clear nailpolish.

Slides were examined under a light microscope using a 10 power objective lens for determination of percent tissue loss. The same tissues described in histochemistry studies were examined for digestion. Micrographs were taken at this time to provide a pictorial representation. Finished slides were stored at 4°C until used for image analysis.

Image Analysis

The previously prepared AP slides were used to measure undigested percent area taken up by each tissue, while the digested slides were used to look at percent tissue loss relative to the undigested sections. A computer-based optical image analyzer was used for the analyses. The hardware and software were run by a Image Build Analysis System (IBAS 2.0, Kontron, West Germany). One section from each slide was viewed under a Zeiss microscope, at 10 X, which was connected with a C-mount to a video camera (Bosch, West Germany) and digital images of the sections were displayed on a video screen. The image was viewed for focusing and correct positioning of the section using the microscope controls. The stem images used were wedge-shaped and extended from the epidermis to the innermost parenchyma. The images were stored on Bernoulli disks

(20 megabyte) until analyzed.

For each image, the area occupied by each tissue was calculated based on the total area of the section. The area measurements were performed by the computer program after the tissue area had been defined manually using a digitizer stylus. Percent area taken up by each tissue was calculated as a percentage of individual tissue area relative to total area. Cell wall length and width were calculated for each tissue from the user-defined area using a binary thinning procedure and measuring the length and width of the thinned line. A pictorial representation of the steps employed in this procedure is included in Appendix 2.

Tissue area was analyzed from the undigested slides. The percent loss due to digestion was based on the percent area difference between the undigested and digested sections for tissues which showed some digestion after 24 hours in rumen fluid. The cell wall width and cell wall length as determined by the program were also analyzed. The number of vascular bundles (VB) appearing in the recorded image was also analyzed to examine for possible variation. Area data was recorded as a percent value and cell length and width were measured in microns.

Enzymatic Dry Matter Digestibility, Neutral Sugar and Phenolic Acid Analysis

The enzyme preparations were provided by J.D. Erfle and all procedures were performed in conjunction with his laboratory (Centre for Food and Animal Research, Ottawa). For clarity, the full procedures used by Dr. Erfle are included in Appendix 3

and major points are summarized below.

Enzymatic Dry Matter Disappearance. A subsample of ground material prepared for the lignin/ADF analysis was subjected to enzymatic hydrolysis for determination of DM digestion. Plant material, buffer and cellulase enzymes were mixed and incubated in a shaking water bath at 48 to 50°C for 16 hours. Tubes and contents were cooled and centrifuged. The supernatant was collected, frozen and saved for phenolic acid and sugar analyzes.

The precipitate was resuspended and rinsed onto filters which were dried. The filters and residue were weighed and a percent dry matter disappearance was calculated.

Estimation of Total Carbohydrates by Acid Hydrolysis. Total sugars present in forage were estimated after hydrolysis with sulphuric acid. Plant material and 72% sulphuric acid were mixed and heated for 1 hour. Deionized water was then added and contents were heated for another hour. After cooling, solid lead carbonate was added to neutralize the solution. The mixture was centrifuged. The supernatant was frozen and stored for the high pressure liquid chromatography procedure (HPLC).

Estimation of Alkaline-labile Phenolic Acids. Forage samples and 0.1 N NaOH were mixed and heated for 20 hours. After centrifugation, 6 N HCl was added to neutralize the solution. The sample was frozen and stored for HPLC analysis.

Estimation of Carbohydrates by HPLC. The supernatant from incubations of forages with fungal enzyme preparations or neutralized sulphuric acid hydrolysed forage samples were used for HPLC analysis. Results were expressed as micromoles/100 mg DM.

Phenolic Acid Estimation by HPLC. The phenolic acids were estimated from the supernatant from the enzyme or alkaline hydrolysis procedures using similar HPLC equipment as for the sugar analysis. Again, results were expressed as micromoles/100 mg DM.

Statistical Analysis

Data collected from the effect of growth stage experiments was analyzed as a split-plot with the genotypes as the main plots and the three stages of growth as sub plots. Effect of genotype was tested against the genotype by replicate interaction error term, while effect of growth stage and growth stage by genotype were tested against the growth stage by replicate within genotype interaction error term. Individual plants were treated as experimental units. As the data was unbalanced (not all 6 plants survived), the General Linear Models (GLM) procedure of SAS (1985) was used.

The lignin and ADF contents, enzymatic dry matter digestibility, phenolic acid and neutral sugar data met the requirements of the GLM procedure. The histochemical data was transformed by square root (x+1), area data was transformed by arcsine (square root (x/100)), vascular bundle number was transformed by ln (x+1) and cell wall data was transformed by 1/(x*x) to achieve homogeneous variances and normal distribution of the data. The cell wall length data was also multiplied by 10^8 to provide useful values. The analysis of variance was performed on transformed data, where applicable, and

untransformed means are presented in the results. Least square mean comparisons were only performed if $P \leq 0.05$. Histochemical data exhibiting genotype differences at $P \leq 0.10$ was considered to be biologically significant due to the subjectiveness of the procedure, but genotype mean comparisons were not performed.

RESULTS AND DISCUSSION

Growth Experiment

Tiller and leaf number prior to harvest for the digestibility experiment were similar among the nine genotypes (Table 1). Growth characteristics such as tiller and leaf production were evaluated to determine if any genetic variation existed for these traits among the 9 genotypes studied. This information also helps to prevent selection of a high digestibility genotype which may have extremely poor growth and yield characteristics (Casler and Hovin 1985). As well, morphological plant characteristics which are associated with quality could serve as indices for quality screening criteria. Sachs and Coulman (1983) noted that a majority of 36 genotypes of RCG tested had relatively uniform, vigorous and tall vegetative growth. Only a few genotypes had stunted growth and late maturity. Frank et al. (1985) did, however, report significant differences among 6 genotypes for rate of leaf emergence. Casler and Hovin (1985) performed an experiment with RCG where they counted the number of tillers, but the authors were developing a prediction equation for yield, and unfortunately did not report on the variation among genotypes for number of tillers. There needs to be more research into tiller and leaf number for RCG genotypes to determine if variation does exist.

TABLE 1. Number of tillers per pot and leaf number of the main stem at harvest for the parent genotypes of Rival z

Genotype	Tiller number	Leaf number
109	13	12
85	12	7
89	12	7
96	12	8
113	11	7
83	10	8
80	10	8
114	10	9
91	8	11
	S.E. 1.07	S.E. 1.09

z Analysis of variance shown in Appendix 4.

Gravimetric Digestibility Analysis

Differences in stem digestibility were observed among genotypes (Table 2). There was a difference of 8 percentage units between the lowest and highest digestibility genotypes (91 and 96). The leaf digestibilities did not show any significant differences among genotypes, despite the greater range (13 percentage units) between the highest and lowest leaf digestibility (genotypes 80 and 113). The difference in digestibility between leaves and stems was approximately 30 percentage units, which was expected based on the results of Norton (1982) who, after studying a variety of forage species, concluded that leaves usually have higher digestibilities than stems at the same maturity. The degree of difference varied with the species and the maturity level.

Since stems, rather than leaves, offered the greater potential for genetic variation, all further studies utilized stem material only. Genotype 109, although it had the second highest stem digestibility, was not chosen since it tended to have weak stems which were prone to bending and breaking. Thus, genotypes 96 and 83 were chosen as the high digestibility material and genotypes 80 and 91 were chosen as the low digestibility material.

There are a variety of possible explanations for the lack of differences among genotypes in leaf digestibility. Leaves may have higher individual variation which would require the use of more plants per genotype to observe statistical differences. The leaf sections also had a lower unit weight than stems sections, and small errors in weighing may cause large errors in the results. Increasing the number of sections taken from one

Table 2. Relative digestibilities of stem and leaf fractions from parent genotypes of Rival using an in vitro gravimetric digestion technique z

Genotype	Stem % digestion	Leaf % digestion
96	31.75 a	58.43
109	30.76 ab	57.01
83	30.44 ab	58.17
85	28.39 bc	54.41
114	27.92 bc	57.81
113	26.64 cd	47.77
89	25.41 cd	53.21
80	24.06 d	59.47
91	23.77 d	54.57
S.E.	1.31	3.08

a-d Means within columns followed by different letters are different by the Student-Newman-Keuls test at $P \leq 0.05$.

z Analysis of variance shown in Appendix 5.

leaf to 15-20 sections, would have increased the mass and may have reduced this type of error. As well, leaves are more fragile than stems and despite careful handling, are more prone to tearing or breaking. This tearing would result in an increase in surface area for the rumen microorganisms to initiate digestion and would provide for higher percent digestion in the same time. The last scenario would only hold true if a 24 hour incubation did not provide sufficient time for maximum leaf digestion. To date, no other reports on RCG have looked at the differences among genotypes using separate forage fractions.

The more mature forage was selected for the gravimetric digestibility study in an attempt to find the greatest differences among genotypes. The six leaf stage was chosen arbitrarily as a compromise between full maturity and time available to complete the experiment. Many species of forage grasses have been reported to show a wider range in digestibility among genotypes with maturity (Schank et al. 1973; Klock et al. 1975; Sleper and Mott 1976); although a few species have also been reported to have similar ranges in genotypic digestibilities in both young and mature forage (Hacker and Minson 1972). The effect of maturity on degree of difference with respect to digestibility has not been examined for RCG, but it was assumed that the use of older material would provide genotypic differences.

Some researchers harvest their experimental material on the basis of age rather than maturity stage. Sampling material on an age basis can add extra problems since some genotypes mature at a faster rate than other genotypes leading to confounding errors. The results may be corrected for the maturity differences, but it is simpler to harvest based

on some maturity stage (Lentz and Buxton 1991).

Lentz and Buxton (1991) reported that total herbage, leaves, stems and sheaths from orchardgrass genotypes with wider leaves consistently had higher digestibilities than those from genotypes with narrow leaves, and that the length and width of leaves were positively correlated with IVDMD. The authors suggested that wider leaves had greater percentages of non-lignified tissues. Buxton and Marten (1989) did not find significant variation for IVDMD among RCG genotypes selected for high or low specific leaf weight. Buxton (1990) reported few significant differences for lignin and NDF with the same plants.

Several authors have reported that variation in stem digestibility accounted for most of the variation in digestibility of total herbage among genotypes (Christie and Mowat 1968; Buxton and Marten 1989; Casler and Carpenter 1989). Hacker (1982) reported that for most species of forage there is less genetic variation in leaf digestibilities than stem digestibilities. Stem material collected from orchardgrass had greater variation among genotypes for lignified area and had more lignified area than leaf or sheath material (Lentz and Buxton 1991). The authors concluded their paper by stating that variation in stem IVDMD was more important than variation in leaf IVDMD for quality improvements in orchardgrass.

Tilley and Terry (1964) reported that stems influenced digestibility to a greater extent with increased maturity than leaves. Hanna et al. (1976) also reported that the reduction in IVDMD with maturity in bermudagrass was primarily due to changes in the digestibility of the stems. Results of the gravimetric digestibility study combined with the

observations reported in other studies were the basis of the decision to do all future work with the stem material.

Stem Lignin and ADF Content

Since sufficient ground material was available, all four replicates were analyzed for ADF and lignin content. Data collected from the analysis indicated differences among genotypes 91 and 96 for both parameters (Table 3). Lignin values for genotypes 80 and 83 were similar to genotype 91 but ADF values were similar to that of genotype 96. The degree of difference among genotypes was greater with lignin than ADF percentages, suggesting that lignin analysis may be of greater value in genotype evaluation programs than ADF analysis if only one analysis can be performed.

Lignin concentration increased ($P \leq 0.01$) and ADF content decreased ($P \leq 0.01$) with increasing growth stages. Interestingly, both ADF and lignin are usually assumed to increase with age (Hacker 1982). The growth stage effects indicate that RCG should be harvested before growth stage 2 for lower lignin content; but if the harvest is taken past growth stage 2 the increase in lignin content will be moderate. For lower ADF percentages, growth stage 2 or 3 would be the best harvest time, but this stage results in higher lignin levels; thus, it appears that growth stage at harvest will be a compromise between higher lignin and lower ADF percentages. Since both lignin and ADF are associated with reduced digestibility (Van Soest 1982), more experimentation is required to determine which characteristic is more successful at reducing digestibility and thus is

TABLE 3. Acid detergent fibre (ADF) and lignin concentations, % DM basis, of stems from RCG genotypes z

Variable	ADF	Lignin
Genotype		
91	37.95 a	4.49 a
80	37.41 a	3.41 b
83	37.33 a	3.42 b
96	35.35 b	3.20 b
Growth Stage		
1	38.43 A	2.92 B
2	37.89 A	3.91 A
3	34.72 B	4.04 A
Growth Stage*Genotype		
	ns	ns

a-b Means within columns followed by different letters are different by the Least Squares Probable Difference test at $P \leq 0.05$.

A-B Means within columns followed by different letters are different by the Least Squares Probable Difference test at $P \leq 0.05$.

ns Not significant

z Values shown represent data means. Analysis of variance shown in Appendix 6.

more essential to forage evaluation programs. Genotypes performed similarly with increasing growth stage.

Although genotype differences relating to digestibility were indicated with the ADF procedure, other authors have not always found a relationship, suggesting that the ADF content may not affect digestibility as much as lignin content does. For example, Vogel et al. (1981) reported significant differences between low and high IVDMD strains of switchgrass, but Anderson et al. (1988) and Gabrielson et al. (1990), using the same strains, were not able to find any differences in forage ADF levels.

Marum et al. (1979) reported variation among 12 RCG genotypes for ADF and lignin content in the whole plant. Buxton (1990) reported that there were no significant differences among several genotypes of RCG for ADF and lignin content. Casler (1990) did not find any significant differences for ADF among 20 genotypes of RCG. Thus, there is still room for further investigations into genotype variation in ADF and lignin percentages in RCG.

No other experiments with RCG have examined the effect of maturity on the ADF percentages. Burritt et al. (1982, 1984) studied the increase in lignin content in RCG over six harvest dates and reported an increase in lignin concentration from harvest 1 to harvest 6; however, the increase was not linear as the authors had expected from research with other species. The current study showed greater increases in lignin deposition from growth stage 1 to growth stage 2 than from growth stage 2 to growth stage 3. Burritt et al. (1982, 1984) analyzed whole forage, whereas the current study

analyzed the stem material only, accounting for some possible discrepancies in data interpretation.

Histochemistry

Section staining for lignin demonstrated differences among genotypes for the epidermis (E), cortex (C), sclerenchyma ring (SR), vascular bundles (VB) and parenchyma (PA) with both stains indicating that these tissues contain both syringyl units and coniferaldehyde units in the lignin. Examples of section staining are shown in Figures 4 and 5.

The E gave low to moderate reactions with the AP stain, with the more intense colouration occurring with the less digestible genotype (Table 4). Staining reactions with CS indicated intense colouration in genotype 91, and weaker but similar colour reactions for the remaining genotypes. Variation in colour reaction due to growth stage was apparent for epidermal tissue stained with AP but not with CS, which suggests the type of lignin compound associated with reduced digestibility due to growth stage or maturity may be different than that associated with genotype variation. Genotypes behaved similarly with increased growth stage.

Akin (1989) reported that the E stained intensely with AP and weakly with CS in cool-season grass stems. It is important to note that the data summarized from earlier research was recorded as strong/intense, weak or none, while the data in this study was rated on a scale of 7, with different colours for the CS and AP stains; thus, it is possible that

Figure 4. Examples of reed canarygrass stem sections (16 microns) stained with acid phloroglucinol (AP).

Examples a, b and c are derived from genotype 91 and sections d, e and f are from 96. Sections a and d are representative of growth stage 1, b and e from growth stage 2 and c and f are from growth stage 3. The increase in intensity of staining with increased growth stage is apparent in genotype 91, but not as apparent from genotype 96. The stronger reactions in the xylem and vascular bundles relative to the other tissues are quite clear. The phloem shows a complete absence of reaction.

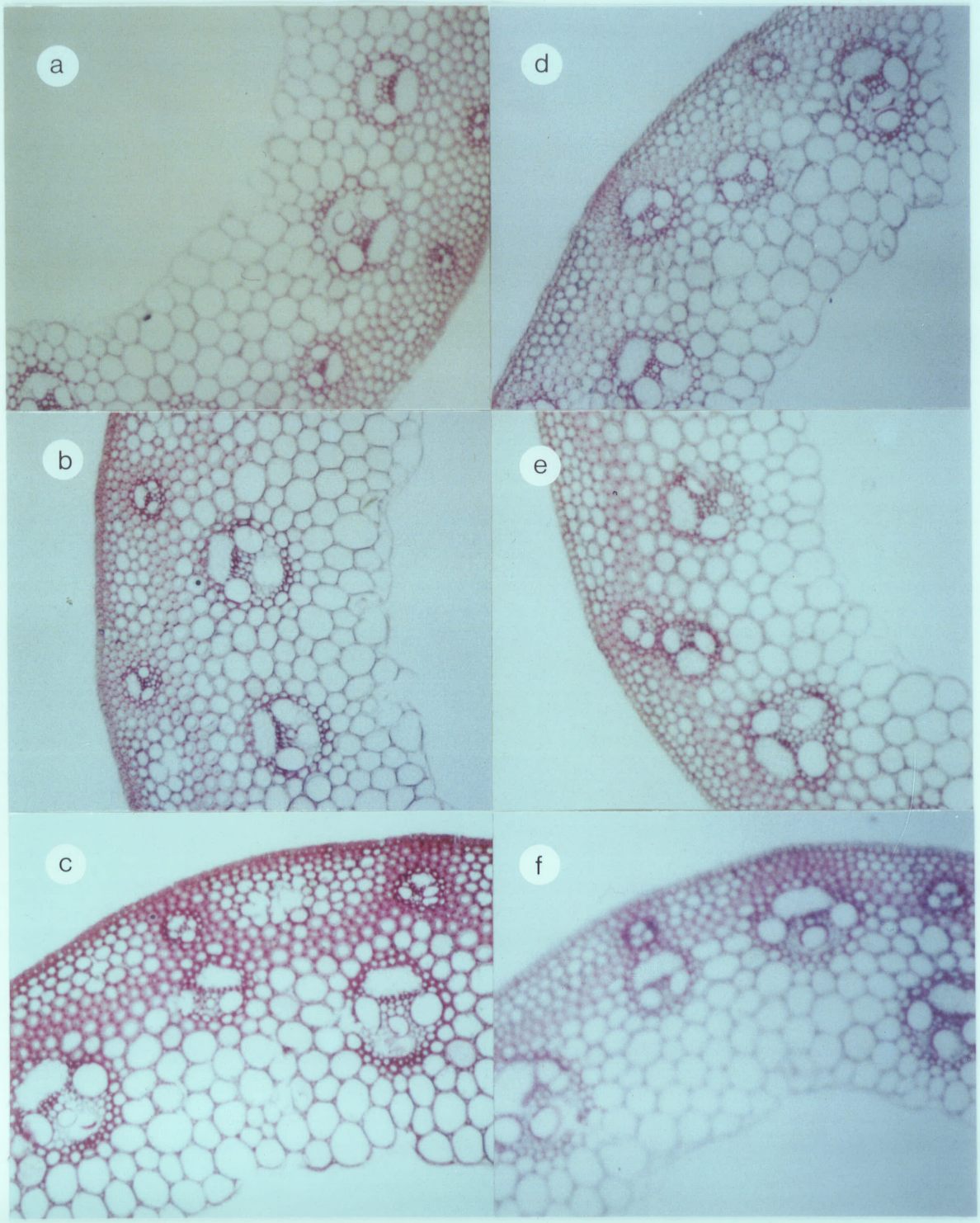

a
d
b
e
c
f

Figure 5. Examples of reed canarygrass stem sections (16 microns) stained with chlorine sulphite (CS).

Sections a, b and c are taken from genotype 91 and sections d, e and f are taken from genotype 96. Sections a and d are from growth stage 1, sections b and e are from growth stage 2 and sections c and f are from growth stage 3. The effects of genotype and growth stage are not as apparent as when viewed directly with a microscope due to the colour filter required for picture taking. However, the intense reaction in the X relative to other tissues can be observed. The difference in reaction colours for CS compared to AP can be seen by comparing the two figures.

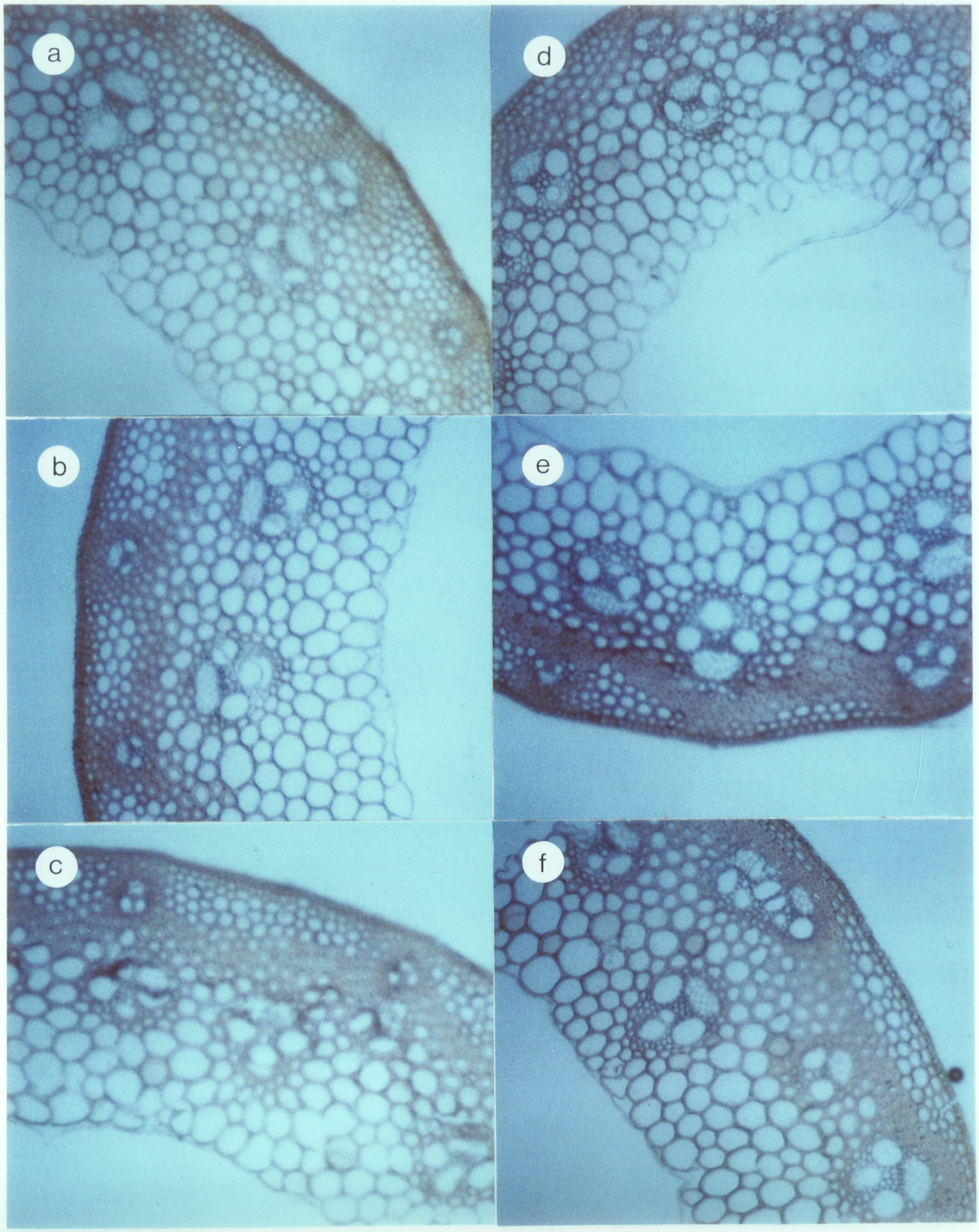

a
d
b
e
c
f

TABLE 4. Means colour scores for the epidermis of RCG stem sections stained with either acid phloroglucinol (AP) or chlorine sulphite (CS) stains z

Variable	AP score	CS score
Genotype		
91	3.5 a	5.3 a
80	2.3 b	5.0 b
83	1.8 bc	5.0 b
96	1.4 c	4.9 b
Growth Stage		
1	1.6 B	5.0
2	2.4 A	5.0
3	2.7 A	5.2
Growth Stage*Genotype		
	ns	ns

a-c Means within columns followed by different letters are different by the Least Square Means Probable Difference at $P \leq 0.05$.

A-B Means within columns followed by different letters are different by the Least Square Means Probable Difference test at $P \leq 0.05$.

ns Not significant

z Analysis was performed on transformed data square root (x+1). Analysis of variance is shown in Appendix 7.

differences in the rating scales may account for the reported weaker reaction in the X with the CS stain. The CS stain does definitely stain less intensely (reddish/orange to orange/yellow) than the AP stain (various shades of red and pink); therefore, if the stains were not rated independently, the CS stain would appear to have weaker reactions than AP. In this data, the CS reaction was actually more intense (within its scale) than the AP reaction, suggesting that there may be a higher proportion of syringyl units to coniferaldehyde units in E lignin than in the other tissues. Goto et al. (1991) reported medium reactions with AP and CS for the E of barley straw stems which support the findings of this experiment.

Genotype differences in staining reaction were also apparent in the C, with the greatest range in reaction between genotypes 91 and 96 (Table 5). For both stains, growth stage 1 provided weaker colour reactions than growth stage 2, but growth stages 2 and 3 had similar reaction intensities. Genotypes 91 and 96 had the lowest CS stain ($P \leq 0.05$) reaction at growth stage 1. By growth stage 2, the colour reaction was greatest ($P \leq 0.05$) for genotype 96, the other genotypes demonstrated more moderate rates of increase. At growth stage 3, the low digestibility genotypes had the highest CS colour reactions. No growth stage*genotype interactions were observed with the AP stain. None of the research previously recorded has looked at the histochemical reaction in the C of stems; however, since all genotypes and cuts stained, this suggests that the cortex in stems is an important tissue as it does contain lignin, and thus may affect the digestibility of the forage.

TABLE 5. Mean colour scores for the cortex of RCG stem sections stained with either acid phloroglucinol (AP) or chlorine sulphite (CS) stains z

Variable	AP score	CS score
Genotype		
91	4.5 a	4.6 a
80	3.7 b	4.6 a
83	3.0 c	4.4 ab
96	2.9 c	4.3 b
Growth Stage		
1	2.1 B	3.9 B
2	4.2 A	4.7 A
3	4.3 A	4.7 A
Growth Stage*Genotype		
	ns	*

a-c Means within columns followed by different letters are different by the Least Square Means Probable Difference test at $P \leq 0.05$.

A-B Means within colomns followed by different letters are different by the Least Square Means Probable Difference test at $P \leq 0.05$.

ns Not significant

* Significant at $P \leq 0.05$

z Analysis was performed on transformed data square root (x+1). Analysis of variance is shown in Appendix 7.

The SR also showed genotype differences ($P \leq 0.05$) with both stains (Table 6), with the greatest range in colour intensity between genotype 91 and genotype 96. Both stains indicated an increase in lignin units with advanced growth stage, with each growth stage being different from the others. The CS stain showed a growth stage*genotype interaction, indicating that the genotypes react differently with increased growth stage in the deposition of syringyl units. For example, genotypes 91 nad 96, which had the lowest initial colour reactions, had a large increase in syringyl units between growth stages 1 and 2, and no increase between growth stages 2 and 3, while genotypes 80 and 83 did not change between growth stages 1 and 2 and had a large increase in syringyl units between growth stages 2 and 3.

Akin (1989) summarized several experiments to report that the SR of cool-season grass stems stained intensely with AP and weakly or no reaction with CS. Again, the scoring systems are likely different. Goto et al. (1991) reported that the SR of barley straw stems gave a medium reaction with both AP and CS which more accurately reflects the data in this experiment.

Genotype differences were observed for the AP staining of the VB, with the greatest range in score between genotypes 91 and 96 (Table 7). A similar trend ($P \leq 0.10$) was observed for CS reactions. Both stains gave scores between 4-6 (medium to high). All growth stages provided different reactions for the AP stain, while the CS stain indicated that growth stage 1 was different from growth stage 2, but growth stages 2 and 3

TABLE 6. Mean colour scores for the sclerenchyma ring of RCG stem sections stained with either acid phloroglucinol (AP) or chlorine sulphite (CS) stains z

Variable	AP score	CS score
Genotype		
91	4.1 a	4.3 a
80	3.3ab	4.2 b
83	2.7 b	4.2 b
96	3.1 b	3.9 c
Growth Stage		
1	2.0 C	3.8 C
2	3.6 B	4.2 B
3	4.2 A	4.5 A
Growth Stage*Genotype		
	ns	**

a-d Means within columns followed by different letters are different by the Least Squares Means Probable Difference test at $P \leq 0.05$.

A-C Means within columns followed by different letters are different by the Least Squares Means Probable Difference test at $P \leq 0.05$.

** significant at $P \leq 0.01$

ns Not significant

z Analysis was performed on transformed data square root (x+1). Analysis of variance shown in Appendix 7.

TABLE 7. Mean colour scores for vascular bundles of RCG stem sections stained with either acid phloroglucinol (AP) or chlorine sulphite (CS) stains z

Variable	AP score	CS score
Genotype		
91	5.5 a	4.7
80	4.9 b	4.5
83	4.7 bc	4.2
96	4.5 c	4.2
Growth Stage		
1	4.4 C	4.0 B
2	5.0 B	4.5 A
3	5.3 A	4.7 A
Growth Stage*Genotype		
	ns	ns

a-c LS means within columns followed by different letters are different by the Least Squares Means Probable Difference test at $P \leq 0.05$.

A-C Means within columns followed by different letters are different by the Least Squares Means Probable Difference test at $P \leq 0.05$.

ns Not significant

z Analysis was performed on transformed data square root (x+1). Analysis of variance is shown in Appendix 7.

provided similar syringyl unit concentrations in the lignin. Increasing growth stage did not affect genotypes differently. The strength of the reactions with both stains used in this study indicated that lignin in the VB contains high amounts of both coniferaldehyde and syringyl units.

Akin et al. (1977) reported that the VB was AP positive, but did not report the CS reaction. Akin et al. (1984) reported that 6 of 7 Panicum species had positive CS reactions for the VB of stems, and all 7 stained positive with AP.

The PA gave similar ranges in colour scores and significance as the E (Table 8). As for all tissues, except for the VB, the CS stain produced a stronger reaction (within its scale) than the AP stain, suggesting that these tissues may have higher proportions of syringyl units relative to coniferaldehyde units. All layers of the PA were stained, even at growth stage 1. The coniferaldehyde units increased in quantity from growth stage 1 to 2, but were not further increased by growth stage 3, while the syringyl units increased with each growth stage. The ranking of the genotypes will vary depending upon which growth stage plants are harvested. For example, the CS staining indicated that the genotypes react similarly at growth stage 1, but at growth stage 2, genotypes 83 and 96 are acting the same as at growth stage 1, and genotypes 91 and 80 are depositing syringyl units, the rate of deposition being greatest for genotype 91. No further deposition of syringyl units was observed for genotypes 91 and 80; however, deposition was observed

TABLE 8. Mean colour scores for the parenchyma of RCG stem sections stained with either acid phloroglucinol (AP) or chlorine sulphite (CS) stains z

Variable	AP score	CS score
Genotype		
91	2.5 a	4.7 a
80	2.3 a	4.4 b
83	2.2 a	4.3 bc
96	1.4 b	4.2 c
Growth Stage		
1	1.0 B	4.0 C
2	2.5 A	4.4 B
3	2.7 A	4.8 A
Growth Stage*Genotype		
	*	**

a-c LS means within columns followed by different letters are different by the Least Squares Means Probable Difference test at $P \leq 0.05$.

A-C Means within columns followed by different letters are different by the Least Squares Means Probable Difference test at $P \leq 0.05$.

* significant at $P \leq 0.05$, ** significant at $P \leq 0.01$

z Analysis was performed on transformed data square root (x+1). Analysis of variance shown in Appendix 7.

for 96 and 83 from growth stage 2 to 3. Growth stage 3 colour reactions were greater fro genotypes 91 and 83 than for the other 2 genotypes. The genotype*growth stage interaction observed with the AP stain was due to an increase in colour reaction for genotypes 91, 80 and 83 from growth stage 1 to 2 with no change across the growth stages for genotype 96.

Akin et al. (1977, 1984) reported that PA from the lower internodes (older material) gave positive reactions for CS and AP, while PA from the upper internodes (young material) did not stain or only the layers of PA nearest the SR or close to the VB stained. These results suggest that in Panicum species and bermudagrass there is an age-related increase in syringyl and coniferaldehyde units of lignin in the PA as evidenced by the increase in intensity of the staining reactions. Akin (1989) reported that mature PA of cool-season grass stems stained intensely with CS and intensely to none with AP. Goto et al. (1991) reported that the PA in one of three barley cultivars had a greater score with AP than the other two, but the CS scores were similar. The authors theorized that there was a difference in the chemical make-up of the PA with all 3 cultivars having similar quantities of syringyl units but one had a greater proportion of coniferaldehyde units than the other 2. The PA of RCG appears to reach maturity, identified as the point where all cell walls are lignified, quite rapidly as all layers stained at growth stage 1.

The X consistently had a colour score of 7, the highest score of any tissue, over all growth stage and genotypes for both stains, thus was not included in the statistical analysis. No other tissue had a colour score of 7, indicating that of the tissues examined, X had the greatest amount of both syringyl units and coniferaldehyde units of lignin. It

can be concluded that since growth stage did not affect the colour score of X, this tissue had already completed lignification by the time the plants reached the 4 leaf stage which was approximately 4 weeks after tiller planting. Stafford (1962) stated that histochemical reactions in the SR rather than in the X may more accurately represent the site of variability in lignification among grass genotypes. This statement is particularly appropriate to RCG, as the X did not show any variability among genotypes or growth stages. Akin et al. (1977) also reported strong positive reactions for the X in bermudagrass. In his review, Akin (1989) summarized earlier research and reported that the X stained intensely with AP in both warm- and cool-season grass stems. However, the CS stain was reported to stain weakly or give no reaction. Again, scoring system differences are likely responsible for the reported weak staining of the X by the CS stain.

The P was the only tissue to show no reaction with the stains and was assigned a score of 0 at all growth stages. This result was expected based on previous research with stem material (Akin et al. 1977, 1984). An absence of response suggests that the P does not contain lignin, or if there is lignin, there are no syringyl or coniferaldehyde units in the lignin.

Overall, both stains produced rankings for the genotypes which were similar to the gravimetric results, with genotype 91 having the highest scores, suggesting that it is the most lignified, and 96 having the lowest scores. The effect of growth stage was significant for both stains in the stem C, SR, VB and PA. The AP staining for the E was significant at $P \leq 0.05$, but the CS staining was significant at $P \leq 0.10$. These findings indicate that both syringyl and coniferaldehyde units increase in quantity in these stem

tissues with increasing growth stage, and are likely the tissues responsible for the increased lignin content seen with the lignin analysis. These data confirm that earlier harvests will result in forage with greater digestibility.

Rate of lignin deposition or type of lignin deposited is not uniform for the above tissues as the plant matures. For example, the syringyl units in the VB begin to plateau at growth stage 2, while the coniferaldehyde units continued to increase up to growth stage 3. Conversely, the syringyl units in the PA continued to increase to growth stage 3, while the coniferaldehyde units did not significantly increase past growth stage 2. In the C, staining reaction strength did not increase past growth stage 2 for either the AP or CS stain. This information supports Stafford's (1962) finding that all functional types of lignin increased in quantity as plants mature, but the rate of increase and/or order of increase was often different among tissues. The results of this study also indicate that the types of lignin, as represented by coniferaldehyde and syringyl units measured in this study, may increase independently of each other.

There were significant growth stage*genotype interactions for the CS staining of the C, SR and PA and for the AP staining of the PA. These findings indicate that the stage of growth of RCG stems will affect the degree of difference among genotypes for the above tissues and stains. Genotypes 91 and 96 had similar deposition of syringyl and coniferaldehyde units in the tissues and with the stains mentioned above at growth stage 1. The C and PA of 91 reached maximum levels of syringyl unit deposition by growth stage 2, while 96 did not obtain maximum levels until growth stage 3. Although the SR of both 91 and 96 reached near maximum levels of syringyl unit deposition by growth

stage 2, the levels achieved by 91 were 0.9 units higher than in 96. The PA of 91 reached maximum levels of coniferaldehyde unit deposition by growth stage 2 as did 96, but the increase in coniferaldehyde unit deposition in 96 was not significant.

Genotype 80 is an interesting case, as it was determined to have low digestibility by the gravimetric procedure, but had relatively low lignin contents when evaluated with the method of Goering and Van Soest (1970). The staining of the C with CS resulted in similar values for both 80 and 91, while the AP staining indicated that 80 was different from the other genotypes. The AP staining of the SR and PA indicated that 91 and 80 had similar levels of coniferaldehyde units. These stem tissues may be key to determining how individual tissues influence digestibility since both low digestibility genotypes had similar values.

From its origins, histochemistry has been criticized because it is only a qualitative tool and not a quantitative tool (Stafford 1962). The author also added that the accuracy of CS is questioned due to the lack of stable colouration over time, and reported that both AP and CS may have variation in colours due to rates of penetration into the tissues. In this experiment, very thin sections of a consistent thickness were used to reduce variation due to the rate of penetration of the stains. The sections were rated within a maximum of 5 minutes after slides were prepared to prevent errors due to colour fading. No incidents of colour fading or variation from section to section on a slide were noticed in this experiment. The use of AP and CS in ranking experiments is thus recommended with the aforementioned precautions.

The subjectivity of histochemistry has also been questioned (Stafford 1962). However,

colour codes were found which provided samples for most of the colours seen with the RCG sections. The use of these codes did reduce the subjectivity of this test. The CS stain may be questioned more strongly due to the apparently conflicting results between the RCG work and the experiments performed by Akin and his group. The different scoring systems may have caused these differences, or RCG may not follow the trends set by warm season grasses and the several cool season grasses examined by Akin. The recently-developed technique of separating the individual tissues and then analyzing them (Grabber and Jung 1991; Grabber et al. 1991) may be useful in settling the differences. If the tissues could be separated and analyzed for the actual concentrations of syringyl and coniferaldehyde units, it could be determined if the positively-reacting tissues, except for the VB, do have greater proportions of syringyl units to coniferaldehyde units, as indicated by the higher scores for the CS stain.

Histochemistry may not be beneficial for all forage evaluation programs as it may not always pick up differences among genotypes, especially in leaves (Ames, personal communication). However, this may only be a problem with materials of high digestibility, as the histochemistry results of the RCG stems demonstrated an ability to establish differences among genotypes and growth stages.

Thus, before implementing histochemistry into a plant breeding program, researchers should try different stains and thicknesses of sections to determine how to obtain easily observable differences. Keeping these possible problems in mind, histochemistry is a quick and simple tool to look at differences among genotypes for potential digestibility and it is recommended to plant breeders as an evaluation technique.

Current histochemical techniques do not provide information on the phenolic composition of the tissue types. The phenolic composition of cell walls is an area of intense research and Akin et al. (1990) suggested that the next phase of research should be to develop stains which stain different colours for specific compounds to efficiently identify the phenolic types.

Section-to-Slide

Complete digestion of stem P was observed in sections from all genotypes and growth stages following the 24 hour rumen fluid incubation period. The PA had variable digestibility with 83 and 96 having the higher disappearance of PA (Table 9). Other tissues did not appear to be digested when observed with a light microscope. Examples of incubated sections are shown in Figure 6.

Although the other tissues did not digest sufficiently to be observed with a light microscope, it is possible that some microbial digestion occurred, releasing some cell wall compounds. This could account for the differences among genotypes detected by the gravimetric digestibility analysis, but not observed in the section-to-slide (STS) digestion procedure.

The use of a scanning electron microscope (SEM) might show this level of digestion. Another method of examining digestion not seen with a light microscope would be to use an enzyme preparation rather than rumen fluid for the incubation medium, and to measure products released by enzymatic activity. The STS has shown genotype

TABLE 9. Percent parenchyma disappearance of RCG stem sections incubated in rumen fluid for 24 hours, as determined with a light microscope z

Variable	Parenchyma digestion (%)
Genotype	
91	2.9 c
80	3.5 bc
83	5.1 ab
96	6.6 a
Growth Stage	
1	8.5 A
2	3.5 B
3	1.6 B
Growth Stage*Genotype	
	ns

a-c Means followed by different letters are different by the Least Squares Means Probable Difference test at $P \leq 0.05$.

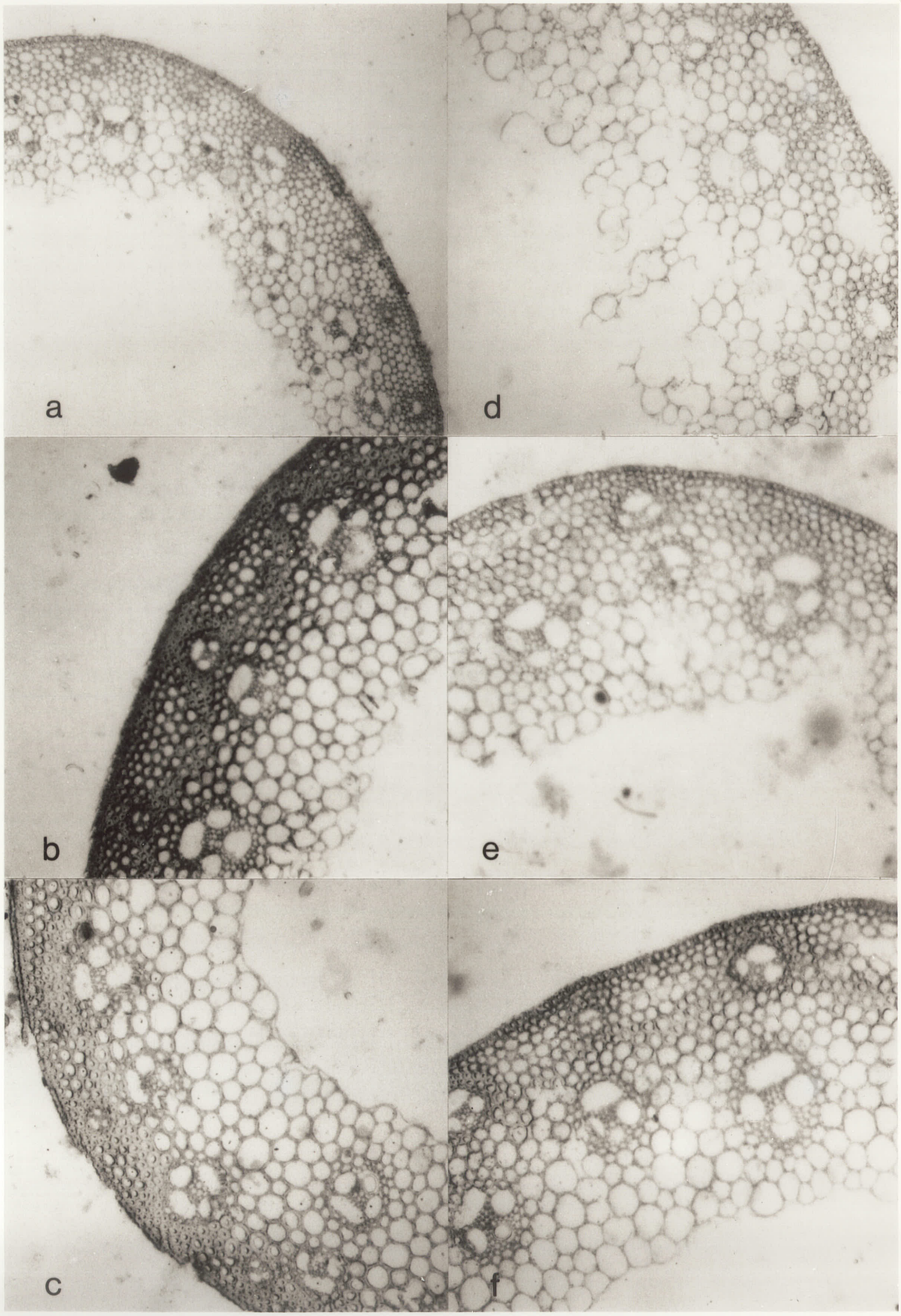
A-B Means followed by different letters are different by the Least Squares Means Probable Difference test at $P \leq 0.05$.

ns not significant

z Analysis was based on transformed data arcsine (square root (x/100)). Analysis of variance shown in Appendix 8.

Figure 6. Examples of reed canarygrass stem cross sections (16 microns) after a 24 hour incubation in rumen fluid.

Sections a, b and c are sections obtained from genotype 91 at growth stages 1, 2 and 3 respectively. Figures c, d and e are sections taken from genotype 96 at growth stages 1, 2 and 3 respectively. Notice the reduced parenchyma digestion with increased growth stage for both genotypes. Both genotypes show obvious digestion at growth stage 1, while at growth stage 2 the digestion is less obvious, especially in 91. At growth stage 3, the parenchyma of both genotypes is vitually fully intact. These micrographs clearly show the genotype and growth stage effects indicated from the analysis of variance of the collected data.


a
d
b
e
c
f

measure products released by enzymatic activity. The STS has shown genotype differences among timothy and switchgrass leaves (Ames, personal communication); therefore, the lack of visually observable digestion may only become a problem with low digestibility material, such as the RCG stems.

There was also a significant ($P \leq 0.01$) growth stage effect with plants at growth stage 1 having higher PA digestion than growth stages 2 or 3. This growth stage effect was also found with the AP stain, but the CS stain indicated that syringyl units increased with each growth stage. The PA digestibility decreased with growth stage in the same manner for all genotypes, although the histochemistry indicated that there were growth stage*genotype interactions. This conflict suggests that although the deposition of coniferaldehyde and syringyl units of lignin increase with growth stage in different patterns in the genotypes, the overall effect of growth stage on the PA digestion is similar for the genotypes. A case in point is seen with 96 and 83. Both had similar CS stain scores at growth stages 1 and 2, but digestibility dropped from 8.5% to 3.5% in the same period.

Other researchers have reported differences in PA digestibility with age for stems. Akin et al. (1977) reported that SEM analysis of sections incubated for 6 hours indicated that the PA in the upper internodes (young material) of bermudagrass was on average 50% digested, while the PA in the lower internodes (older material) was still intact. After 72 hours of incubation, the SR, VB and E were intact in both ages of stems, while the PA was completely digested in the upper internodes, but only slightly digested in the lower internodes, which relates to the results of this research as stem material was

derived from the 1st internode. Using several species of Panicum, Akin et al. (1984) reported similar results. Twidwell et al. (1991) reported that in young switchgrass stems after 48 hours in rumen fluid that the E, VB, X, and SR were 100% intact and the PA was 82% digested. Akin (1989) stated that immature PA was rapidly digested, while middle-aged PA had slow to partial digestibility. Given the results of the STS for this study, the PA of RCG appears to be mature, as determined by reduced digestibility, by growth stage 1, corresponding to stage 14 of Simon and Parks (1981).

Image Analysis

Percent Area

Image analysis of undigested stem sections revealed genotypic differences in the area of the SR, P and PA as a percentage of total stem cross section area (Table 10). Genotype 91 always had different percentages than 96. The percentages of P and PA, considered to be of high digestibility, were greater in 96, while the percent of SR, low digestibility or indigestible, was greater in 91. These findings support the use of percent tissue area in evaluation programs. The E, VB, X and C area percentages were similar across genotypes (Table 11).

Ehlke and Casler (1985) reported that low IVDMD genotypes of smooth bromegrass contained different percentages of SR, PA and X from high IVDMD genotypes, supporting the RCG findings. Goto et al. (1991) reported no significant differences

Table 10. Percent area of phloem (P), sclerenchyma ring (SR) and parenchyma (PA) in RCG stem sections, as determined by image analysis z

Variable	P (%)	SR (%)	PA (%)
Genotype			
91	2.30 b	19.52 a	49.13 b
80	2.01 c	18.37 a	52.94 a
83	2.30 b	17.94 a	52.68 a
96	2.63 a	15.82 b	54.37 a
Growth Stage			
1	2.22	16.43 B	53.46
2	2.37	18.79 A	51.55
3	2.26	18.31 A	51.99
Growth Stage*Genotype			
	ns	ns	ns

a-c Means within columns followed by different letters are different by the Least Squares Means Probable Difference test at $P \leq 0.05$.

A-B Means within columns followed by different letters are different by the Least Squares Means Probable Difference test at $P \leq 0.05$.

ns Not significant

z Analysis was performed on transformed data arcsine (square root (x/100)). Analysis of variance is shown in Appendix 9.

TABLE 11. Mean percent area of the epidermis (E), cortex (C), xylem (X) and vascular bundle (VB) in RCG stem sections, as determined by image analysis z

Variable	E (%)	C (%)	X (%)	VB (%)
Genotype				
91	3.58	10.17	2.54	12.75
80	3.62	9.04	2.32	11.68
83	3.59	8.81	2.44	12.23
96	3.31	8.85	2.56	12.52
Growth Stage				
1	3.68	9.65	2.45	12.12
2	3.49	9.16	2.40	12.24
3	3.39	8.85	2.56	12.53
Growth Stage*Genotype				
	ns	ns	ns	ns

ns Not significant

z Analysis was based on transformed data arcsine (square root (x/100)). Analysis of variance shown in Appendix 9.

among cultivars of barley straw for the percent area of PA, E, SR and VB, which included X and P, in stems. To date other data on genotype or cultivar variation for tissue percentages have not been reported. Several researchers have reported on the percentage of various tissues in stems of warm-season grasses, but did not analyze for genotype or cultivar variation (Akin et al. 1977; Akin et al. 1984; Akin 1989).

An effect of growth stage was observed only for the SR, suggesting that the area of this tissue increases with maturity from growth stage 1 to growth stage 2, but that it does not change significantly with further growth stage increases. The areas of other tissues did not change with growth stage. No growth stage*genotype interactions were indicated.

Schank et al. (1973) reported that limpograss (Hemarthria altissima, Poir) stems had an increase in lignification of the VB but no increase in VB area with maturity. Hanna et al. (1976) found some variation in bermudagrass for the combined areas of VB and SR in stems with increasing age. Akin et al. (1977) did not find any effect of maturity for the percentages of PA, SR, VB or P in stems of bermudagrass. Akin et al. (1984) looked at the percent areas in upper and lower internodes of Panicum species, but did not analyze the data for variation between the maturity levels. More research is needed to determine if the SR (or any other tissues) do increase in area with increases in growth stage in RCG.

Cell Wall Length and Width

Cell wall (CW) length and width of individual tissues were measured on stem sections to examine if differences in these parameters among genotypes reflected digestibility differences. Cell wall length (Tables 12-14) and width (Tables 15-16) of examined tissues were similar across genotypes. While the CW width was fairly constant among tissues, the CW length varied considerably. This is due, in part, to CW length (total length of the thinned line from IA) being measured from the total area of the tissue rather than a constant size of area, thus some of the variation in the values among tissues is due to larger area percentages of some tissues, such as the PA.

Plant maturation resulted in increased CW wall length of the SR. However, the CW width of the SR did not change. More plants per genotype at each growth stage may be necessary to observe differences. The IA program may also be revised such that the thickness of the cell wall may be measured. No cut*genotype interactions were indicated, thus growth stage affected the genotypes in a similar manner.

Hanna et al. (1976) reported that the percent of indigestible anatomical components (VB-P and SR) had a two-fold increase between one and four weeks of age and concluded that the increase was due to an increase in CW thickness (diameter of the CW) in the older material. Unfortunately, the IA program designed for collection of the RCG data was not able to measure the actual thickness of the CW; however, visual observations of the sections did not suggest a major difference in CW thickness among

TABLE 12. Mean cell wall lengths (microns) of the epidermis (E), cortex (C) and phloem (P) tissues of RCG stem sections as determined by image analysis z

Variable	E	C	P
Genotype			
91	1471.62	3123.66	495.65
80	1322.82	2700.56	425.82
83	1320.70	2521.80	490.67
96	1239.40	2864.73	531.28
Growth Stage			
1	1283.51	2864.73	531.28
2	1316.74	2838.42	553.56
3	1408.94	2841.59	492.83
Growth Stage*Genotype			
	ns	ns	ns

ns Not significant

z Analysis was based on transformed data (1/(x*x)). Analysis of variance is shown in Appendix 10.

TABLE 13. Mean cell wall lengths (microns) of xylem (X) and sclerenchyma ring (SR) of RCG stem sections as determined by image analysis z

Variable	X	SR
Genotype		
91	224.30	7026.88
80	166.24	6127.75
83	138.09	6081.47
96	182.13	5499.91
Growth Stage		
1	137.27	5261.43 B
2	188.84	6621.37 A
3	207.38	6583.13 A
Growth Stage*Genotype		
	ns	ns

A-B Means within columns followed different letters are different by Least Squares Means Probable Difference test at $P \leq 0.05$.

ns Not significant

z Analysis was based on transformed data (1/(x*x))* 100000000. Analysis of variance is shown in Appendix 10.

TABLE 14. Mean cell wall lengths (microns) of the vascular bundles (VB) and parenchyma (PA) of RCG stem sections as determined by image analysis z

Variable	VB	PA
Genotype		
91	4547.89	9609.92
80	4169.15	8910.43
83	4038.98	9048.46
96	4377.37	9656.65
Growth Stage		
1	3985.87	8776.10
2	4367.09	9355.58
3	4501.54	9800.31
Growth Stage*Genotype		
	ns	ns

ns not significant

z Analysis was based on transformed data (1/(x*x))* 100000000. Analysis of variance is shown in Appendix 10.

TABLE 15. Mean cell wall widths (microns) of the epidermis (E), cortex (C), phloem (P) and xylem (X) of RCG stem sections as determined by image analysis z

Variable	E	C	P	X
Genotype				
91	1.09	1.24	1.04	1.02
83	1.09	1.10	1.04	1.02
80	1.08	1.13	1.04	1.01
96	1.08	1.10	1.04	1.01
Growth Stage				
1	1.08	1.12	1.03	1.01
2	1.08	1.10	1.04	1.01
3	1.09	1.21	1.04	1.02
Growth Stage*Genotype				
	ns	ns	ns	ns

ns Not significant

z Analysis was based on transformed data (1/(x*x)). Analysis of variance is shown in Appendix 11.

TABLE 16. Mean cell wall widths (microns) of the sclerenchyma ring (SR), vascular bundle (VB) and parenchyma (PA) of RCG stem sections as determined by image analysis z

Variable	SR	VB	PA
Genotype			
91	1.11	1.10	1.10
80	1.11	1.11	1.10
83	1.11	1.10	1.10
96	1.10	1.10	1.10
Growth Stage			
1	1.10	1.10	1.10
2	1.11	1.10	1.10
3	1.11	1.11	1.10
Growth Stage*Genotype			
	ns	ns	ns

ns Not significant

z Analysis was based on transformed data (1/(x*x)). Analysis of variance is shown in Appendix 11.

the tissues. Goto et al. (1991) reported that the CW thickness of PA and E cell walls were different for one of three cultivars, but the cultivar which was significantly different for the PA was not the one which was significantly different for the E. The lack of differences among RCG genotypes for CW width and length, suggest that the differences in digestibility may be related to differences in the biochemical nature of the cell walls, such as lignin or carbohydrate composition.

Vascular Bundle Number

The number of vascular bundles were similar across genotypes and growth stages (Table 17). Genotypes did not behave differently for the growth stages. Schank et al. (1973) had reported variation for VB number in limpograss; however, Akin and Burdick (1973) and Hanna et al. (1976) did not find differences in the number of VB in stems of bermudagrass genotypes. Interestingly, given the apparent difference in digestibility between stems and leaves and the indigestibility of the VB, Hanna et al. (1976) also reported that the percent area of VB was similar for leaves and stems. Ehlke and Casler (1985) discovered variation for VB number in smooth bromegrass stems. From the data in this experiment and previous reports, the variation in the number of VB appears to be species specific, some species of forage grass may have genotypic variation in VB number while other species may not.

TABLE 17. Vascular bundle number determined from RCG stem sections used for image analysis z

Variable	Vascular bundles
Genotype	
91	6.2
83	4.9
80	5.5
96	5.5
Growth Stage	
1	5.9
2	4.5
3	5.2
Growth Stage*Genotype	
	ns

ns Not significant

z Analysis was based on transformed data ln(x+1). Analysis of variance is shown in Appendix 12.

Goto et al. (1991) observed that large VB were surrounded by PA, while smaller VB were located in the SR in barley straw stems. This alignment of large and small VB was also observed in this research.

Digested Section Area

After 24 hours in undiluted rumen fluid, the P was completely digested in all genotypes and all growth stages. The area of the P has already been examined for differences among genotypes.

The PA varied in extent of digestion, thus only the percent area of the PA was examined by IA and the resulting value was subtracted for the undigested section area to give a percent loss due to rumen fluid incubation (Table 18). The majority of calculated values for percent PA digestion following the 24 hour incubation in rumen fluid were negative. Because of the negative values this data was not statistically analyzed. Since the sections were cut from the same stem piece it is extremely unlikely that the digested sections had more PA than the undigested sections. One variable between the undigested sections (AP stained) and the digested sections was that the digested sections were placed on double-sided tape, while the AP sections were put directly on microscope slides. It is possible that the tape held the sections more

Table 18. Percent parenchyma disappearance in RCG stem sections after 24 hour rumen fluid incubaton, as determined by image analysis, by subtraction of digested parenchyma area from undigested parenchyma area z

Genotype	Rep	Growth Stage	Undigested	Digested	Difference
91	3	1	49.04	47.66	1.38
80	3	1	47.32	54.94	-7.62
83	3	1	49.36	52.54	-3.18
96	3	1	53.91	51.54	2.37
91	3	2	53.36	60.85	-7.49
80	3	2	53.25	59.94	-6.69
83	3	2	52.41	53.55	-1.14
96	3	2	55.83	57.10	-1.27
91	3	3	52.13	55.42	-3.29
80	3	3	53.98	44.99	8.99
83	3	3	57.13	57.96	-0.83
96	3	3	51.90	56.53	-4.63
91	4	1	53.44	48.84	4.60
80	4	1	45.61	55.26	-9.65
83	4	1	50.16	56.13	-5.97
96	4	1	58.26	54.30	3.96
91	4	2	47.76	56.03	-8.27
80	4	2	52.00	54.43	-2.43
83	4	2	53.37	54.02	-0.65
96	4	2	49.80	53.28	-3.48
91	4	3	52.30	57.44	-5.14
80	4	3	53.30	50.77	2.53
83	4	3	55.03	54.94	0.09
96	4	3	54.46	57.56	-3.10

z Values shown represent means of the 5 or 6 plants/genotype/replicate.

accurately such that they represented the stem piece, while the sections for AP staining were lifted out of position, as Tissue-Tek is soluble in water-based fluids. Thus, when the stain was drained off, the sections may have been compressed from their original alignment resulting in an apparently reduced area of the tissues, especially the PA. The PA is largely composed of large, thin-walled cells which could be slightly compressed without being noticed. Another experiment should be carried out on a few samples with sections placed on tape and placed directly on microscope slides to see if the taped sections provide higher percentages of PA. Another possibility is that the sections incubated in rumen fluid swelled due to the uptake of the fluid, thus increasing the percent area of the tissues. Again, the PA is probably the tissue most likely to be affected. The values shown in the table were derived from all plants in the genotype/growth stage combinations to reduce potential problems due to the unsticking of tape pieces from the slides and being put on the wrong slides. By using the means of the plants, this problem is avoided.

Enzymatic Dry Matter Digestibility, Neutral Sugar and Phenolic Acid Results

Dry matter digestibility using enzymatic digestion was based on stem material at all three growth stages. The DM digestibilities were similar among genotypes (Table 19). Digestibilities were not affected by growth stage. Genotype 83 had a reduction in DM digestibility from growth stage 1 to 2, but had an increase between growth stage 2 and 3. The DM digestibility of the other genotypes did not vary with growth stage.

TABLE 19. Mean dry matter digestibility of ground RCG stems incubated in a prepared enzyme mixture for 16 hours z

Variable	Digestibility (%)
Genotype	
91	43.5
80	43.6
83	43.3
96	45.0
Growth Stage	
1	44.2
2	42.3
3	44.9
Growth Stage*Genotype	*

* Significant at $P \leq 0.05$

z Analysis of variance is shown in Appendix 13.

Carlson et al. (1969), Hovin et al. (1976), Fairey (1985) and Ostrem (1988) reported values of 62.7%, 65.2, 63% and 72.0% respectively for IVDMD using whole RCG forage and the technique developed by Tilley and Terry (1963). The values reported for this experiment are lower than the other authors, but again only stems were used to determine the values.

Only stem material from growth stage 2, corresponding to stage 16 of Simon and Parks (1981), was subjected to enzyme and acid or alkaline hydrolysis. The neutral sugar (Table 20) and phenolic acid (Table 21) contents released by enzyme incubation were similar among genotypes indicating that the genotypes have similar bonding among moieties. The sugar content released by acid hydrolysis (Table 22) and phenolic acid contents released by alkaline hydrolysis (Table 23) were also similar among genotypes indicating that the genotypes have similar carbohydrate type and quantity. The ratios of FA:PCA did not vary among genotypes.

Using the method of sugar isolation developed by Theander and Aman (1980), Aman and Lindgren (1983) reported values for arabinose, xylose, galactose and glucose for whole forage of RCG over 2 cuts (15 days apart). The authors reported that the sugars were not significantly affected by growth stage. In this experiment only material from growth stage 2 was analyzed. The values reported by Aman and Lindgren (1983) were consistently lower than the values found for this experiment, but as they used whole forage and stems only were used for this experiment, the values seem to be within reason for this experiment.

TABLE 20. Neutral sugar content of RCG stems released by 16 hour incubation in a prepared cellulase mixture z

Sugar	Genotype	Level (umoles/100mg DM)
Arabinose	91	5.56
	80	5.50
	83	5.09
	96	6.36
Fructose	91	15.58
	80	14.26
	83	14.04
	96	17.10
Galactose	91	2.12
	80	2.05
	83	2.20
	96	2.18
Glucose	91	37.97
	80	38.43
	83	39.87
	96	43.70
Xylose	91	17.37
	80	18.32
	83	19.17
	96	20.02

z Analysis was based on untransformed data. Analysis of variance is shown in Appendix 14.

TABLE 21. p-Coumaric acid (PCA), ferulic acid (FA) and FA:PCA content of RCG stems released after a 16 hour incubation in a prepared cellulase mixture z

Variable	Genotype	Level (umoles/100mg DM)
p-Coumaric acid	91	0.31
	80	0.34
	83	0.34
	96	0.38
Ferulic acid	91	0.43
	80	0.39
	83	0.43
	96	0.53
FA:PCA	91	1.40
	80	1.13
	83	1.27
	96	1.37

z Analysis of variance is shown in Appendix 15.

TABLE 22. Neutral sugar content of RCG stems released by acid hydrolysis z

Variable	Genotype	Level (umoles/100mg DM)
Arabinose	91	16.43
	80	14.70
	83	14.21
	96	13.70
Fructose	91	19.24
	80	18.25
	83	15.38
	96	13.29
Galactose	91	6.00
	80	5.77
	83	5.20
	96	4.77
Glucose	91	177.28
	80	174.82
	83	173.49
	96	169.24
Xylose	91	94.58
	80	93.51
	83	92.19
	96	91.13

z Analysis of variance is shown in Appendix 16.

TABLE 23. p-Coumaric acid (PCA), ferulic acid (FA) and FA:PCA content of RCG stems released by alkaline hydrolysis z

Variable	Genotype	Level (umoles/mg DM)
PCA	91	2.25
	80	2.24
	83	2.22
	96	2.00
FA	91	1.05
	80	1.00
	83	0.96
	96	0.90
FA:PCA	91	0.47
	80	0.45
	83	0.45
	96	0.43

z Analysis of variance is shown in Appendix 17.

Besides being of obvious nutritional value, the neutral sugar content of forage is also used as an indication of digestibility. The glucose released by the enzymatic or chemical analysis is used as an indication of cellulose content, since cellulose consists of glucose units (Hatfield 1989). Xylose content is usually considered to be an indication of the xylan content of the hemicellulose, and the galactose content is considered to relate to the pectin content of forages. Both xylose and arabinose are reported to link hemicellulose with lignin (Hatfield 1989). Xylans also tend to be highly substituted with arabinose (Buxton and Brasche 1991). As detailed in the literature review, the contents of cellulose and hemicellulose, as well as their interactions with other cell wall constituents, will affect forage digestibility.

Burritt et al. (1982, 1984) and Bohn and Fales (1991) measured the levels of PCA and FA in whole RCG forage. The values derived from alkaline hydrolysis appear to be reasonable, given that only stems were analyzed in this experiment. Burritt et al. (1982, 1984) measured the PCA and FA levels in RCG genotypes over 6 harvest dates and reported that PCA had a continuous rise over all harvests while FA increased but levelled off towards the last harvest and its rise was not as rapid or continuous. Due to time constraints, only growth stage 2 was analyzed in this experiment so no growth stage effect could be examined. Reed canarygrass at other growth stages should be analyzed to determine if differences exist among genotypes at earlier or later growth stages.

As examined in the literature review, there have recently been investigations into the effects of PCA and FA on forage digestibility. Burritt et al. (1984) reported that the PCA was negatively correlated with IVDMD, but there was not a significant correlation

between FA and IVDMD. Free PCA inhibited the growth and motility of cellulolytic and xylanolytic bacteria, while FA had little effect (Akin 1982b). These results suggest that the PCA content is associated with reduced digestibility, while FA appears to have little or no effect. However, since there were no differences among genotypes in this experiment, phenolics do not appear to influence digestibility of RCG at growth stage 2, corresponding to stage 16 from Simon and Park (1981).

GENERAL DISCUSSION

The first objective of these experiments was to determine if any differences in digestibility existed among the 9 parental genotypes used in the production of Rival using the in vitro gravimetric digestibility technique. Once the genotypes had been narrowed down to two low and two high digestibility genotypes, the other experiments were undertaken to establish stem components contributing to variation in digestibility and to determine alternative methods for collecting information derived from the gravimetric analysis. For example, genotypes 91 and 80 should show characteristics indicative of low digestibility, while genotypes 96 and 83 should show high digestibility characteristics.

The findings of the lignin and ADF analysis indicate that the cultivar Rival may be able to be further improved if genotype 91 was removed from the breeding program. The removal of this genotype may serve to reduce the lignin and ADF concentrations in Rival, thus leading to increased digestibility of the forage.

Generally, the histochemical results followed the same trends as the gravimetric digestibility results, as genotype 91 consistently had the highest colour scores, indicating high lignification, and genotype 96 always had lower scores, indicating low lignification. These two genotypes were always different from each other, except for the CS staining of the VB which was different at $P \leq 0.10$, but still followed the pattern of the other tissues. The two other genotypes were less consistent in their placement, but usually 80 had higher scores than 83 as expected, since 80 had lower gravimetric digestibility than

83. As discussed in the histochemistry section of the results and discussion, the C, SR and PA may be the key tissues which reflect variability in digestibility, since these tissues showed different ratings for the low digestibility genotypes than for the high digestibility genotypes. The AP staining of these tissues was more useful than the CS stain, as only the CS staining of the C showed similar ratings for 80 and 91.

While the STS digestion technique did not show any differences among genotypes, it is possible that digestion was occurring but not at a level sufficient to be observed with a light microscope and this technique may not be useful for low digestibility material. The PA did show some digestion which was different among genotypes with 96 being more digested than 91, but the values were very low compared to those which would be expected from the gravimetric digestibility values. While actual values are not important, the STS values ranged from 10% digestion to 0%, and with such small numbers, the error in estimation is much greater; hence, this technique may not be useful with low digestibility materials.

The areas of the P and the PA as a percentage of total area of stem cross sections were determined to be different among genotypes by image analysis, with genotype 96 having significantly more of each than the other genotypes. The SR also showed differences with 91 having the most area. Akin and Burdick (1975) theorized that differences in the percent area of rapidly digested tissues, such as the P and PA when immature, would affect the rate of digestibility, and that high percentages of these tissues would provide for increased digestibility. If the results of the STS had followed this theory, there should be greater digestion of the P and PA if it could have been seen with a light microscope.

The use of a scanning electron microscope may be beneficial in further studies. The reverse of the hypothesis could also be true. For example, since genotype 91 had the greatest amount of SR, which has low digestibility or is indigestible, this genotype should be more difficult to digest. Both of these theories, if true, would provide for the observed differences seen among the genotypes with the gravimetric digestibility analysis.

The histochemistry analysis showed strong differences between genotypes 91 and 96 for the PA and SR. The CS staining for the SR indicated that both genotypes reach near maximum levels of syringyl unit deposition by growth stage 2, although 96 has only 0.4 units more than at growth stage 1. Both stains with the PA indicated that 91 reached near maximum levels of coniferaldehyde and syringyl unit deposition by growth stage 2, while 96 did not reach maximum levels until growth stage 3. The SR was the only tissue which had similar area percentages for both 91 and 80 suggesting that it might be the tissue whose percent area most affects digestibility. Both hypotheses discussed above thus seem to hold true, and the SR and PA therefore, appear to be the most important tissues in digestibility studies with RCG stems. However, until these results are supported with other species of forage grass, all tissues should be considered to be relevant indicators of digestibility.

As detailed in the literature review, tissues which react positively with AP are usually considered to be indigestible, while tissues reacting positively with CS are partially digestible (Akin and Burdick 1981; Akin et al. 1984; Akin 1989). This does not seem to hold true for RCG, as all tissues, except the P, stained positively with both stains. It is interesting that the PA stained with CS indicating partial digestibility but also stained

with AP supposedly indicating indigestible tissues. The VB, SR, C and E which were not digested, stained with AP as well as CS. The data mentioned above was collected from warm-season grasses, while RCG is a cool-season grass; thus, there may be differences in the chemistry of these grasses which affect the histochemical reactions. All tissues which reacted with the stains, except for the VB and X, had higher scores for the CS than the AP stain. This result suggests a higher proportion of syringyl to coniferaldehyde units of lignin in these tissues. If the above discussion holds true for cool-season grasses, it is possible that these stems may have higher digestibilities than if they contained a greater proportion of coniferaldehyde to syringyl units of lignin.

Both the histochemistry and the digestibility of the PA with the section-to-slide technique indicated significant growth stage differences. This suggests that the PA becomes less digestible with increased growth stage. The SR also showed a significant growth stage effect with the histochemistry and the area of the SR also had a significant growth stage effect, suggesting not only that the SR becomes less digestible with age, but that the area of the SR becomes larger leading to an even greater reduction in potential digestibility. Thus, again the PA and SR seem to be the key tissues involved with reduced digestibility. As well, both the lignification of the SR and PA, and tissue area of the SR are increased with growth stage. Since the gravimetric digestibility analysis was performed only on one cut, these suggestions currently can not be further supported with in vitro digestibility results.

Since the enzmatic digestibility results did not indicate differences among genotypes while the rumen fluid digestibility results did indicate strong differences among the

genotypes, it may be possible that the enzyme technique is not as sensitive as the rumen fluid technique or that the type of enzyme preparation required may be species specific. However, several authors have reported good correlations between rumen fluid and enzyme incubations (Van Soest 1982; Bughrara and Sleper 1986); thus, the lack of differences among genotypes with the enzymes may be an artifact of low digestibility material, such as the RCG stems.

Despite reports of phenolic acids, especially PCA and FA, being involved with the digestibility of forages (Burritt et al. 1982, 1984; Akin 1982b), the results of the RCG work do not appear to support these results. It may be that the levels are not sufficiently high to have an effect at growth stage 2. The results of growth stage 1 or 3 may have shown a relationship with the gravimetric digestibility results.

The results of this work demonstrate that histochemistry, section-to-slide digestion and image analysis techniques can detect differences among plant genotypes and that these differences relate to differences observed for plant digestibility. Many other techniques have been reported to provide differences only among forage species, cultivars or distinct lines of a species bred for digestibility differences.

The techniques described herein also can provide specific information when determining those plant characteristics affecting digestibility. Traditional methodologies providing information on fibre or lignin content provide only general guidelines; however, they cannot provide specific data about tissues key to forage digestion and the relative changes associated with tissue size or composition. This knowledge of tissues is required to understand how plant breeders improve digestibility. Results of this thesis

suggest caution when developing selection programs for improved digestibility. Plant breeders must determine the optimum growth stage and avoid confounding results due to genotype by growth stage interactions.

Near infrared analysis is often cited as being the most valuable tool to plant breeders; however, it still is being improved. As with most commonly used techniques, NIR requires large sample sizes. Increased usage of the technique is based upon improvements in laboratory techniques and improved NIR hardware. As well, NIR only measures bonds in forages, allowing the user to estimate content of specific organic constituent such as ADF or NDF, which are located in many tissues and, therefore, are under multiple gene control. The technology is not specific to certain tissues. Both histochemistry and image analysis procedures can be performed fairly rapidly. The section-to-slide digestion requires approximately 24 hours of rumen fluid incubation, but after the incubation is finished, the results can be rapidly calculated.

All three procedures described herein are inexpensive to perform once the equipment is in place. The image analysis system, while installation cost is high expensive to put in place, requires only a technician and electricity to operate.

SUMMARY AND CONCLUSIONS

The gravimetric digestibility technique showed strong genotypic differences for stem material but not for leaves.

The AP staining of the SR and PA, and both stains with the C, indicated similar values for both of the low digestibility genotypes. The percent area of the SR was also similar for both low digestibility genotypes. These tissues also had growth stage effects for the histochemistry and the SR had a growth stage effect for percent area. Thus, these techniques and these tissues may be the keys to determining digestibility differences among genotypes of forage grasses and examining how digestibility declines with growth stage. Low digestibility genotypes had lower percent areas of P and PA, and higher percent area of SR, thus the percent area of these tissues may be important in forage breeding programs.

The low digestibility genotypes were, in general, characterised by higher levels of syringyl units in the E, C, SR and PA. Coniferaldehyde units in the VB were also higher for the low digestibility genotypes. Conversely, high digestibility genotypes generally had lower levels of syringyl and coniferaldehyde units of lignin. While these tissues did not always show differences between the low and high digestibility genotypes, they did provide genotypic differences and should be considered as important tissues in forage breeding programs.

The lignin and ADF contents and the section-to-slide digestibility values were different between the two low digestibility genotypes, although they did indicate genotype

differences. Vascular bundle number, neutral sugar and phenolic acid contents, and enzymatic dry matter digestibility values did not provide genotype differences.

Histochemistry findings showed strong growth stage effects for most tissues, while the SR was the only tissue whose percent area indicated a growth stage effect. The section-to-slide digestion procedure and the ADF/lignin contents also showed growth stage effects. Thus, these techniques are useful when examining the effect of growth stage on lignin content and reduced digestibility.

There were a number of growth stage*genotype interactions, especially in the histochemistry. Therefore, depending on the technique used, it may be important to harvest the genotypes at more than one growth stage in order to find the greatest difference among genotypes.

The use of histochemistry and image analysis are both recommended to plant breeders for quick and easy evaluation of forage genotypes. Based on the results of these experiments, both biochemical and microanatomical/physical characteristics affect the digestibility of forages and should be evaluated in breeding programs. All tissues examined in this thesis are likely important to digestibility evaluations either positively or negatively and should be considered in selection programs, although the SR and PA may be the key tissues, but further investigation is required before concentrating on only specific tissues.

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APPENDIX 1.
DIETS OF ANIMALS USED FOR THE COLLECTION OF RUMEN FLUID USED IN EXPERIMENTS.

APPENDIX 1.1: DIET OF SHEEP FROM WHICH RUMEN FLUID WAS COLLECTED FOR THE GRAVIMETRIC STUDY.

Ingredient	Percent of Ration
Chopped Grass Hay	30
Grass Haylage	69
Vitamin/Mineral Mix	1

Average Daily Intake = 3-4 kg

APPENDIX 1.2: DIET OF COW FROM WHICH RUMEN FLUID WAS COLLECTED FOR THE SECTION-TO-SLIDE DIGESTION.

Ingredient	kg per day
Corn silage	15
Alfalfa haylage	4
Grass hay	2
Dairy concentrate	6

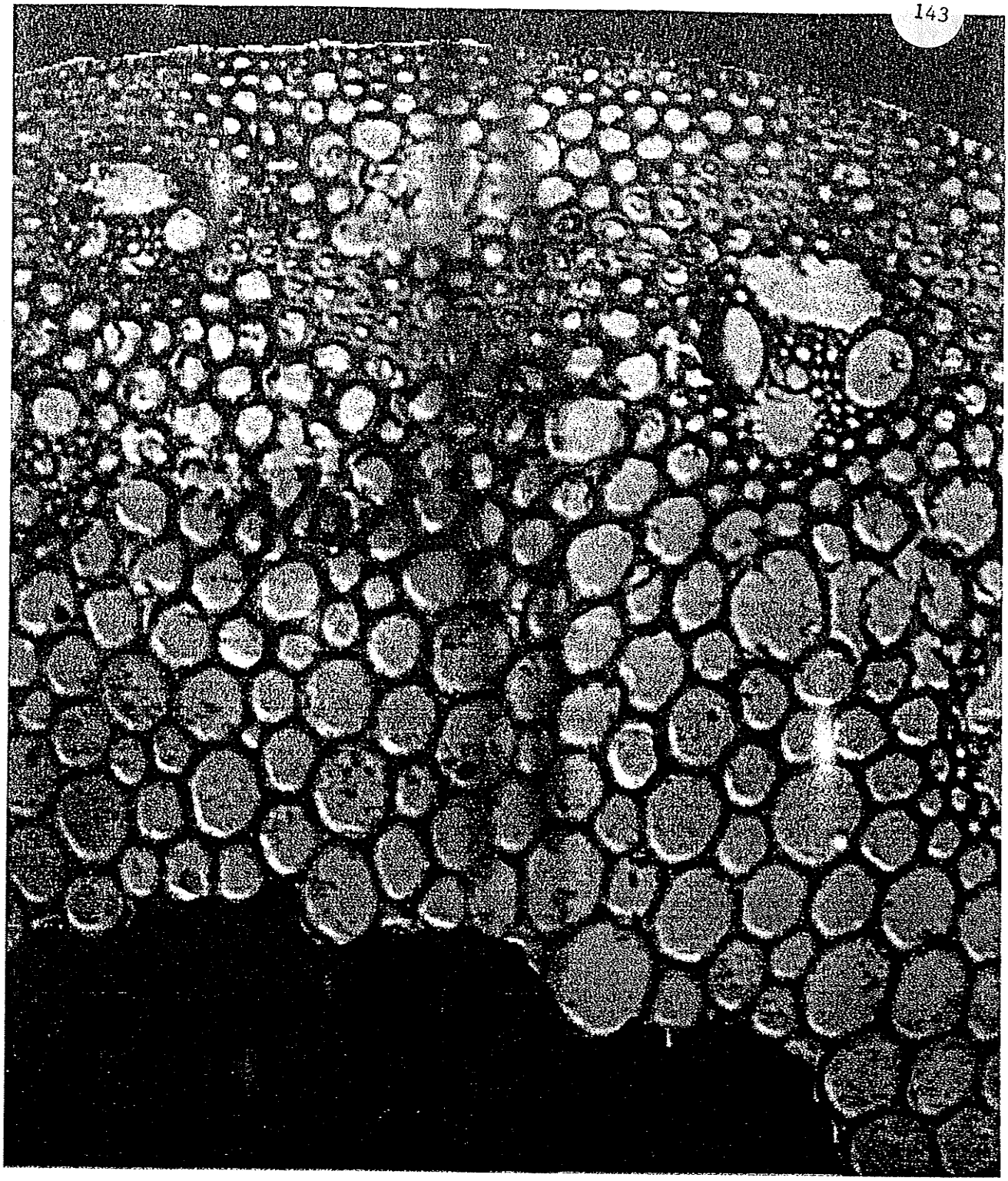
Formulation of Dairy Concentrate

Ingredient	percent on DM basis
Soy-48	39.7
Soybeans	20.0
Ground corn	21.0
Animal fat	5.0
Limestone	4.4
Salt Co-I	3.3
Vitamin/mineral mix	6.2

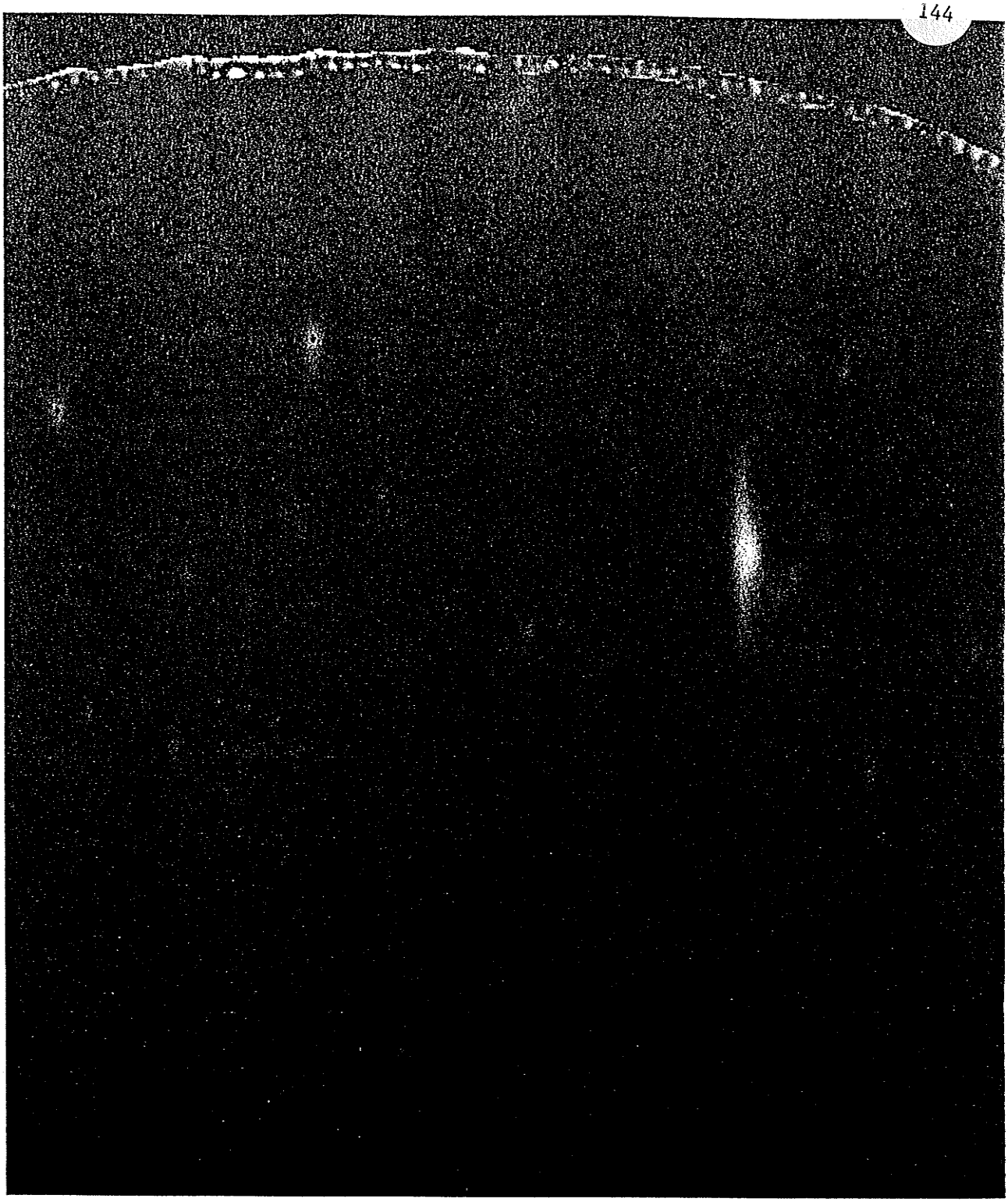
APPENDIX 2.

PICTORIAL EXAMPLES OF

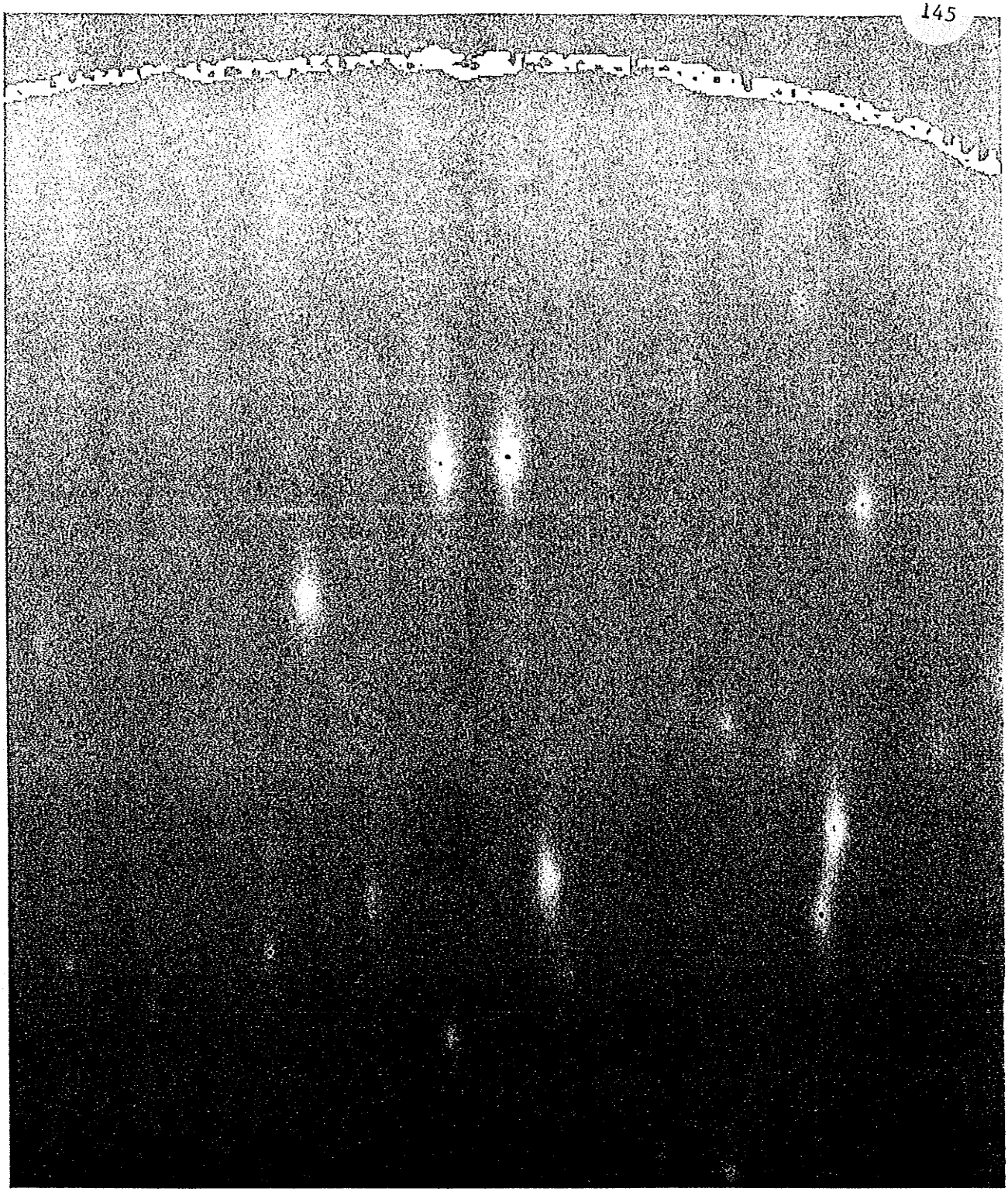
THE IMAGE ANALYSIS PROCEDURE



An example of a reed canarygrass stem cross section (16 um) used for image analysis with unwanted area deleted. The area of this image will be used to calculate total area which is used to determine percent area of individual tissues.

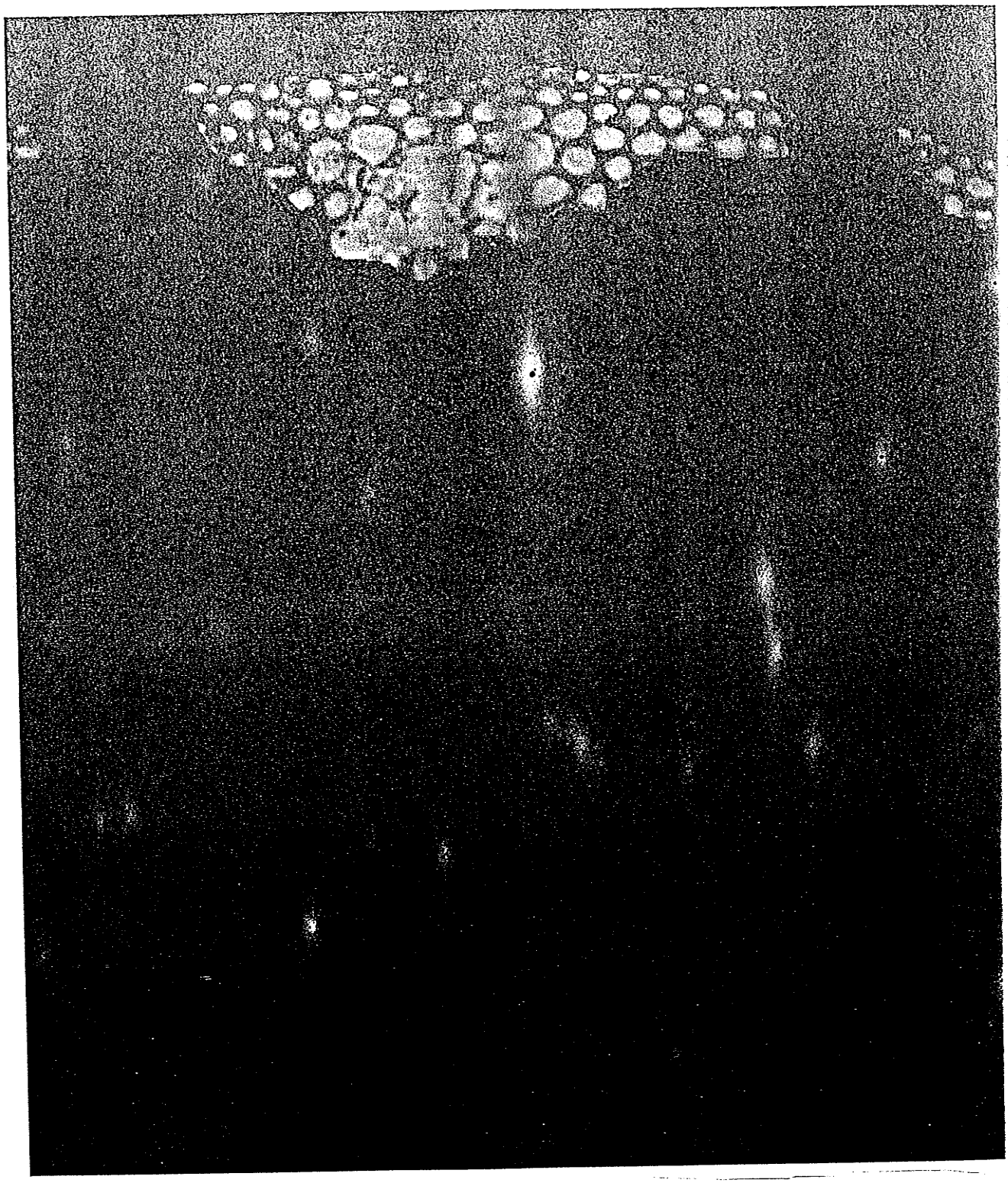


The epidermis of the section after being outlined manually. The program will measure the area of this tissue and express it as a percent based on the total area of the section.

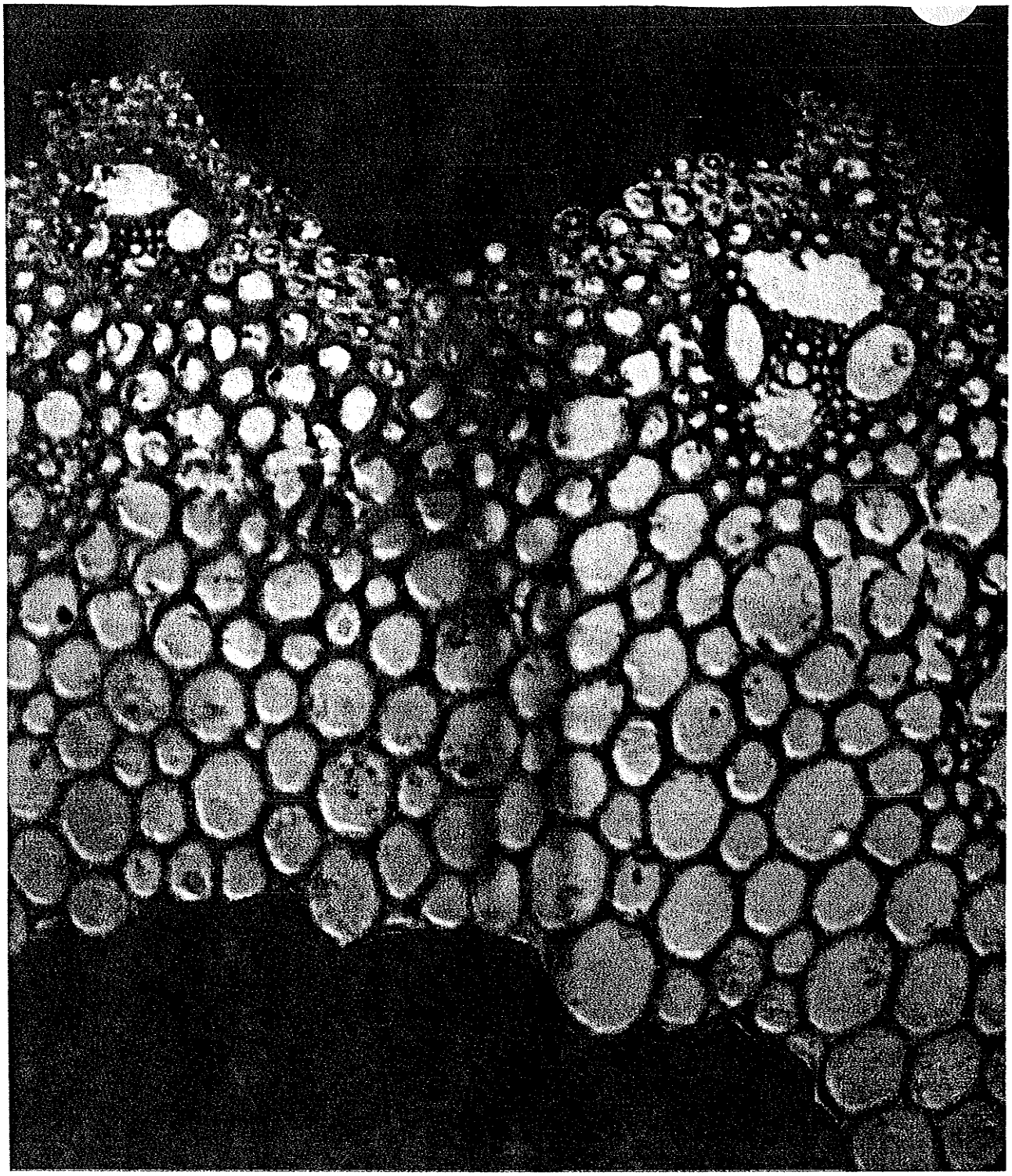


The epidermis after the program has selected the cell wall area of the image. The cell wall area can be adjusted by the operator if needed. Basically, the program deletes the grey areas within the tissue (cell contents) and defines the remaining area as cell wall.

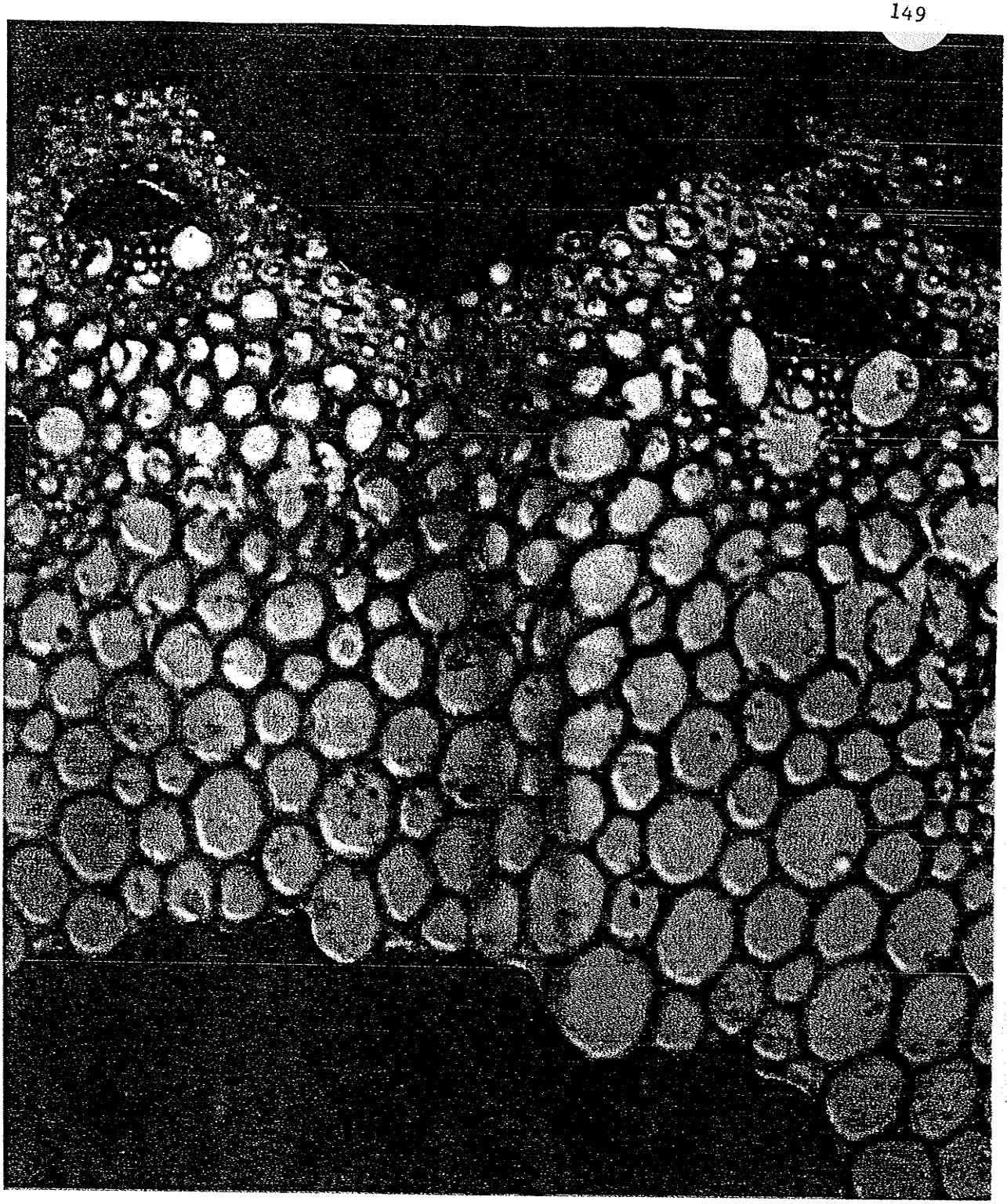
The cell walls of the epidermis after the binary thinning procedure. The program will measure the width of this line and the length of the line after all the pieces are measured to give a total length.



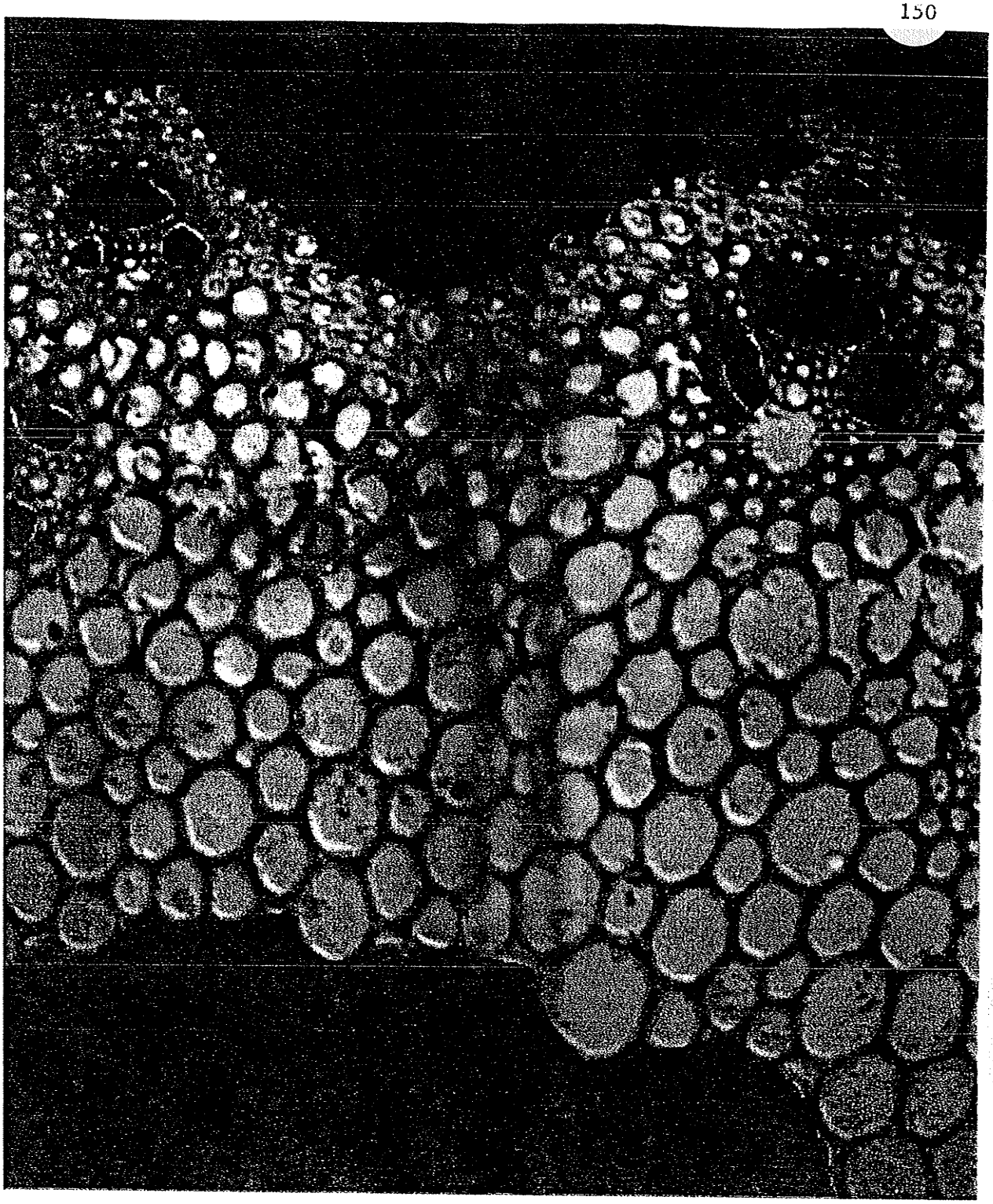
The cortex of the image defined by the operator. Again, the program will measure the area of this image and express it as a percent of the total area of the section.



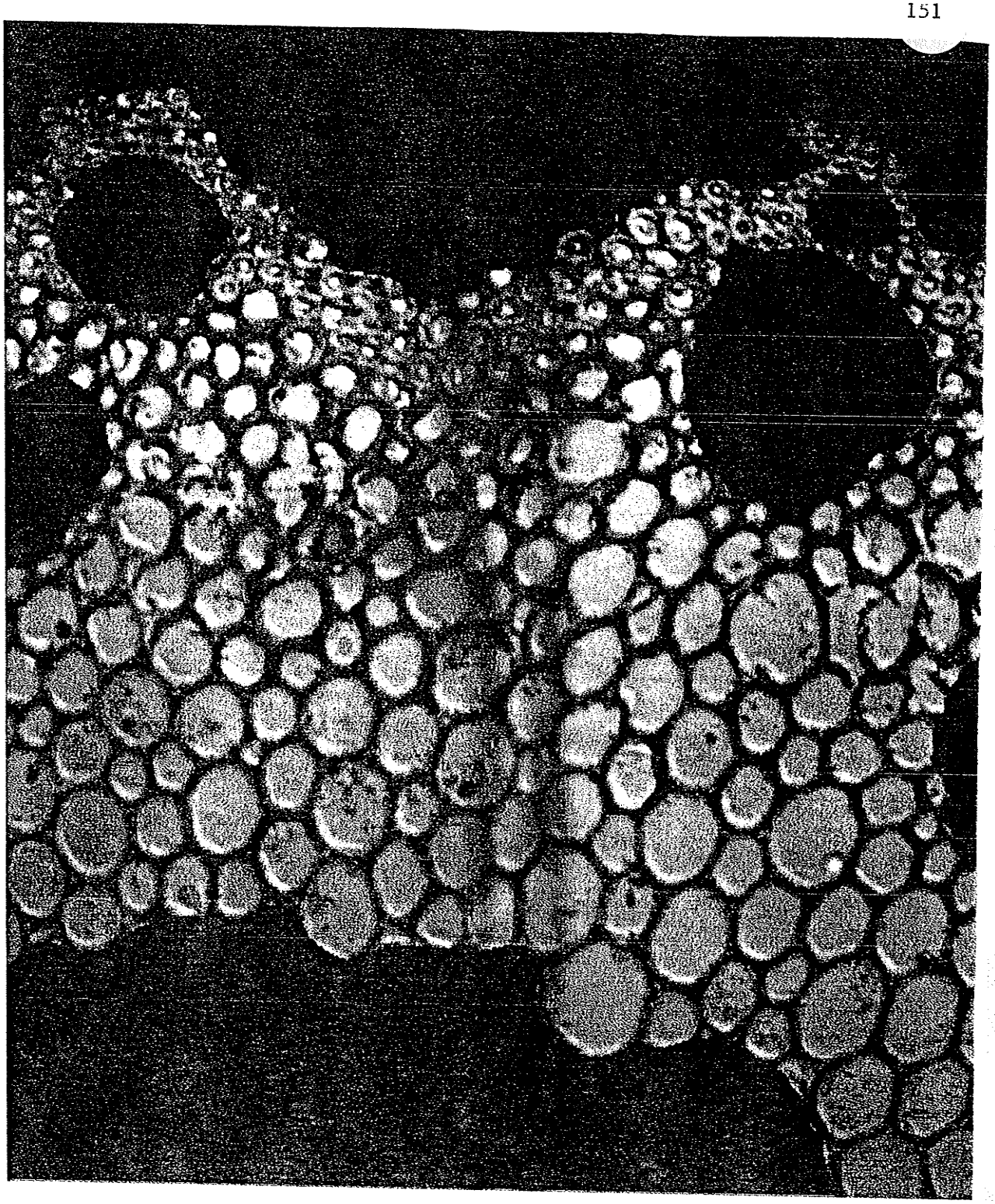
The original image after both the epidermis and the cortex have been defined and eliminated. The program will repeat the cell wall measurement for the cortex as shown with the epidermis.



The image shown previously with the phloem measured and eliminated.

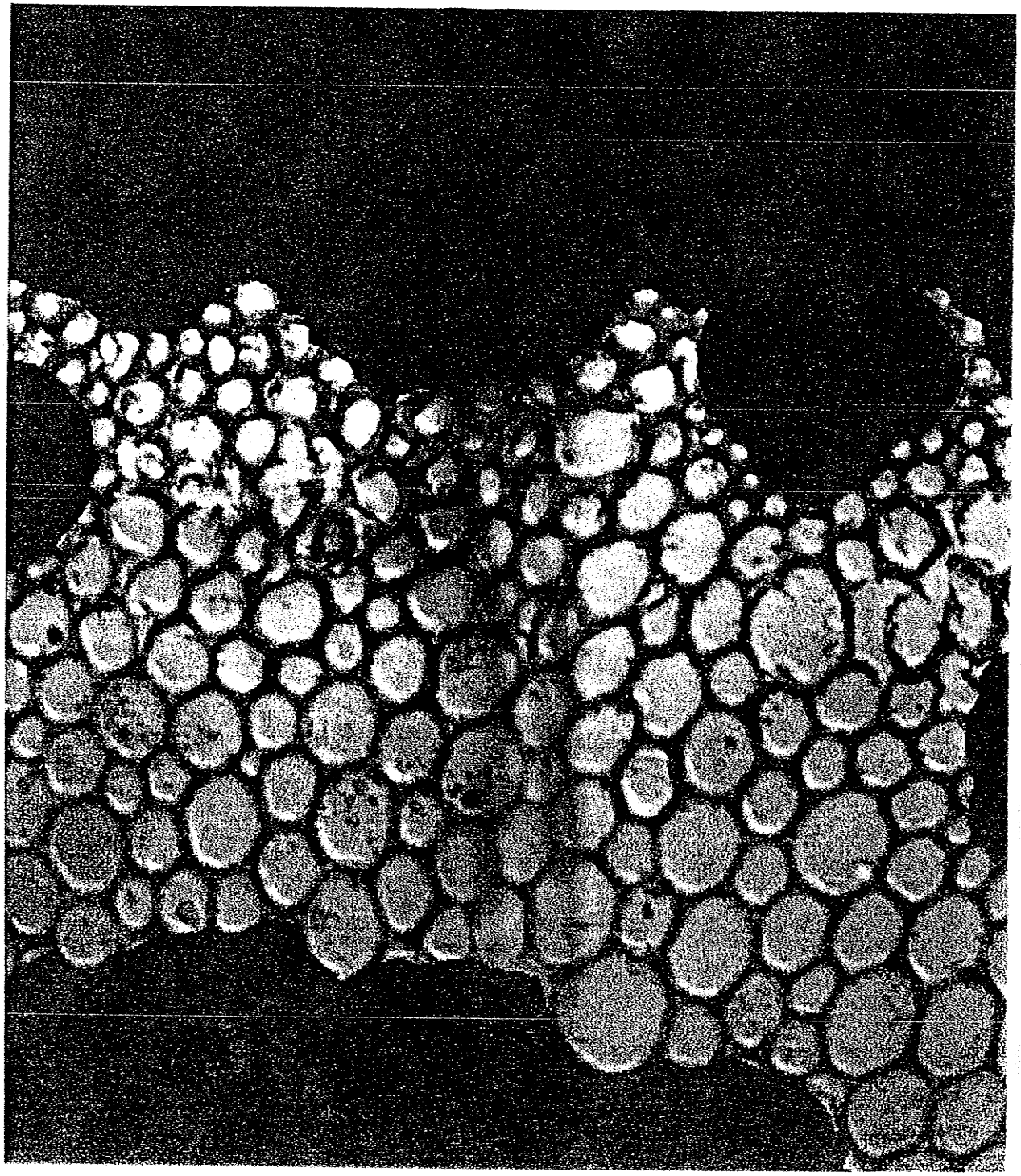


The image shown previously but with the xylem measured and eliminated.



The image shown previously with the vascular bundles measured and eliminated.

The image shown previously with the sclerenchyma ring measured and eliminated, such that only the parenchyma remains. The program will measure the parenchyma and express it as a percentage of the total area of the section as done for all the other tissues. As well, the length and width of the cell wall area after binary thinning is also measured as for the other tissues.



APPENDIX 3.
METHODS FOR DRY MATTER DIGESTIBILITY, PHENOLIC ACID AND NEUTRAL SUGAR CONTENT IN FORAGES.

Enzyme Preparation. Commercial cellulase preparations (Sigma Chem.. Co. St. Louis MO) from Aspergillus niger (200,000 units), Trichoderma viride (20,000 units) and Penicillium funiculosum (400,000 units) were dissolved in 1 to 2 litres of 25 mM citrate-phosphate buffer pH 5.0. The volume was reduced to approximately 100 ml on a Minitan tangential flow concentrator (Millipore Ltd.) and the solution was dialysed on the concentrator against several litres of the same buffer and stored at -75°C until used. Prior to use, the enzyme preparation was assayed using milled filter paper, carboxymethyl cellulose, Oat glucan and oat xylan and laminarin (Erfle, personal communication) to provide activity to hydrolyse 200 to 300 umoles per hour of reducing sugar.

Dry Matter Disappearance. A subsample of ground material prepared for the lignin/ADF analysis was subjected to enzymatic hydrolysis for determination of DM digestion. Samples of 25 mg were weighed into 12 ml serum bottles. Buffer (0.5 ml, 0.1 M citrate-buffer, pH 5.0), fungal enzymes (0.4 to 0.6 ml necessary to provide required activity), 0.1 ml of 0.5% sodium azide, water to total volume of 2 ml were added and the vials sealed. Enzyme preparations were filter sterilized through a 0.22 um sterile membrane filter before adding to incubations. Vials and their contents were incubated in a shaking water bath at 48 to 50°C for 16 hours. After incubation vials were autoclaved at 121°C for 15 minutes to stop the reaction. After cooling the contents were transferred

with two rinses (total of 3 ml) of 0.02% sodium azide to 14 ml plastic centrifuge tubes and centrifuged at 12,000 x g for 10 minutes (Sorval SS34 rotor). The supernatant was removed and saved (frozen) for carbohydrate and phenolic acid analysis.

The precipitate was resuspended in 0.02% sodium azide solution and filtered through Whatman glass microfibre filters which had been predried at 100°C for 12 to 18 hours and weighed on a 12 port sampling Manifold (Millipore Ltd.). After washing the residue on the filters several times they were dried at 100°C for 24 to 48 hours and placed in a desiccator. The glass microfibre filters with the residue were weighed and per cent dry matter disappearance calculated.

Estimation of Total Carbohydrates by Acid Hydrolysis. Total sugars present in forage were estimated after hydrolysis with sulphuric acid according to the method of Sloneker (1971). The forage sample (25 mg of dry material) was weighed into a 15x125 mm Teflon-lined screw-capped test tube. Sulphuric acid (0.3 ml of 72% w/w) was added to each test tube and the tube contents heated at 30°C for 1 hour. To disperse material in sulphuric acid, contents of tubes were stirred with a glass rod and a Vortex mixer. The glass rod was broken and the bottom portion left in the tube. After 1 hour of heating the contents of the tubes were diluted with 8.4 ml of deionized water and the diluted samples placed in a preheated autoclave and further heated at 120°C for 1 hour. Tubes were cooled, centrifuged and a 6 ml aliquot was neutralized by the addition of solid lead carbonate. The precipitated lead sulphate was removed by centrifugation and the supernatant stored frozen for carbohydrate analysis by the HPLC method outlined below.

Estimation of Alkaline-labile Phenolic Acids. The method of Borneman et al. (1990) was used. Dry forage samples (100 mg) were weighed into Teflon capped test tubes and 8 ml of 0.1 N NaOH were added. The tubes were gassed with nitrogen and heated for 20 hours at 20°C. The suspension was centrifuged and a 5 ml aliquot was neutralized with 6 N HCL. The total volume was recorded and samples were stored frozen until phenolic acids were analyzed by HPLC as described below.

Estimation of Carbohydrates by HPLC. The supernatant from incubations of forages with fungal enzyme preparations or neutralized sulphuric acid hydrolysed forage samples were centrifuged or filtered (0.45 nm filter) into HPLC vials for carbohydrate analysis. The column used was a Dionex Carbopak PA1 column with a PA guard column. A 15 mM NaOH solvent was passed through the column at a flow rate of 1 ml/minute for 18 minutes followed by regeneration for 10 minutes with 250 mM NaOH and then equilibrated with 15 mM NaOH for 15 minutes. The column was integrated into a Waters HPLC system with two Model 510 pumps, a WISP 710b sample injection unit all controlled by a Model 840 chromatography data station. The sugars were estimated using a Dionex Pulsed Amperometric Detector (PAD, Dionex Canada Ltd.) with a gold PAD cell filled with 300 mM NaOH. Solvents were degassed with helium using a Dionex Eluant Degas Module. To maintain detection sensitivity 600 mM NaOH was added post column with a Dionex Reagent Delivery Module at flow rate of approximately 0.3 ml/minute. Solvents also were degassed prior to entering the pumps with an Erma Inc. ERC 3320 Model in-line degasser. The PAD was set with voltages for E1=0.05, E2=0.65 and E3=-0.95. The response time was 1 second, the range was set at 1.0 and

the output at 3nKAFS. The column was run at ambient temperature. Standard sugars (20 nmoles each arabinose, galactose, glucose, xylose, fructose xylobiose and cellobiose) were injected in a volume of 10 ul. Sample volume injected varied with time of incubation and total incubation time.

Phenolic Acid Estimation by HPLC. The phenolic acids were estimated from the supernatant using the same HPLC equipment used for carbohydrates except that a Spherisorb-ODS1 column (5 um, 4.6 x 2500 mm operated at 50°C) was used and detection was with a Waters 490 Programable Multiwavelength Detector using the Maxplot feature to scan for peaks at 308 and 322 nm to detect p-coumaric and ferulic acids respectively. The solvent used was 2.5% n-butanol and 0.3% acetic acid in deionized water at a flow rate of 2 ml/minute. Standard phenolic acids (2 nmoles each of p-coumaric and ferulic) were injected in 10 ul for quantitation and results were expressed on the basis of 100 mg of dry matter.

APPENDIX 4.
ANALYSIS OF VARIANCE OF TILLER NUMBER AND LEAF NUMBER FOR THE PARENT GENOTYPES OF RIVAL.

4.1: Analysis of Tiller Number

Source	DF	Type III SS	F Value	Pr > F
GENO	8	64.06592222	1.75	0.1379
REP	3	22.52247500	1.64	0.2064
ERROR	24	109.84070000		

4.2: Analysis of Leaf Number

Source	DF	Type III SS	F Value	Pr > F
GENO	8	96.92250000	2.24	0.1371
REP	3	3.23744444	0.23	0.8774
ERROR	24	114.65405556		

APPENDIX 5.
ANALYSIS OF VARIANCE OF LEAF AND STEM DIGESTIBILITIES FOR PARENT GENOTYPES OF RIVAL.

5.1: Analysis of Leaf Digestibility

Source	DF	Type III SS	F Value	Pr > F
GENO	8	1272.003149	1.84	0.0903
REP	3	2465.447139	9.49	0.0001
GENO*REP	24	2732.407769	1.31	0.2003
GENO*PLANT	16	1721.456123	1.24	0.2683
ERROR	56	4676.557819		

Tests of Hypothesis using GENO*REP as an error term

Source	DF	Type III SS	F Value	P > F
GENO	8	1272.003149	1.40	0.2481

5.2: Analysis of Stem Digestibility

Source	DF	Type III SS	F Value	P > F
GENO	8	826.2356461	13.47	0.0001
REP	3	148.7846396	6.47	0.0008
GENO*REP	24	496.1377538	2.70	0.0013
GENO*PLANT	16	161.5589154	1.32	0.2214
ERROR	56	414.10354335		

Test of Hypothesis using GENO*REP as an error term

Source	DF	Type III SS	F Value	P > F
GENO	8	826.2356462	5.00	0.0010

APPENDIX 6.
ANALYSIS OF VARIANCE OF STEM ADF AND LIGNIN VALUES FOR SELECTED RCG GENOTYPES.

6.1: Analysis of ADF Values

Source	DF	Type III SS	F Value	Pr > F
GENO	3	92.3038564	137.95	0.0001
REP	3	77.6941049	116.11	0.0001
GENO*REP	9	21.3755494	10.65	0.0001
CUT	2	249.9517308	560.32	0.0001
CUT*GENO	6	36.1941632	27.05	0.0001
CUT*REP(GENO)	24	260.4296991	48.65	0.0001
ERROR	46	10.2600000		

Tests of Hypotheses:

a) using the Type III MS for GENO*REP as an error term

Source	DF	Type III SS	F Value	Pr > F
GENO	3	92.30385641	12.95	0.0013

b) using the Type III MS for CUT*REP(GENO) as an error term

Source	DF	Type III SS	F Value	Pr > F
CUT	2	249.9517308	11.52	0.0003

c) using the Type III MS for CUT*REP(GENO) as an error term

Source	DF	Type III SS	F Value	Pr > F
CUT*GENO	6	36.19416323	0.56	0.7607

6.2: Analysis of Lignin Values

Source	DF	Type III SS	F Value	Pr > F
GENO	3	23.12846731	65.55	0.0001
REP	3	14.22000000	40.30	0.0001
GENO*REP	9	2.43506327	2.30	0.0315
CUT	2	22.03808894	93.69	0.0001
CUT*GENO	6	0.46363839	0.66	0.6843
CUT*REP(GENO)	24	7.30060185	2.59	0.0028
ERROR	46	5.41000000		

Tests of Hypotheses:

a) using the Type III MS for GENO*REP as an error term

Source	DF	Type III SS	F Value	Pr > F
GENO	3	23.12846731	28.49	0.0001

b) using the Type III MS for CUT*REP(GENO) as an error term

Source	DF	Type III SS	F Value	Pr > F
CUT	2	22.03808894	36.22	0.0001

c) using the Type III MS for CUT*REP(GENO) as an error term

Source	DF	Type III SS	F Value	Pr > F
CUT*GENO	6	0.46363839	0.25	0.9528

APPENDIX 7.
ANALYSIS OF VARIANCE OF THE HISTOCHEMISTRY OF RCG STEM SECTIONS STAINED WITH EITHER ACID PHLOROGLUCINOL (AP) OR CHLORINE SULPHITE (CS).

7.1: Analysis of AP Results for the Epidermis

Source	DF	Type III SS	F Value	Pr > F
GENO	3	5.97320268	4.81	0.0001
REP	1	0.14689695	2.57	0.1121
GENO*REP	3	0.12447093	0.73	0.5391
CUT	2	2.18064589	19.06	0.0001
CUT*GENO	6	0.73851151	2.15	0.0535
CUT*REP(GENO)	8	0.59521366	1.30	0.2513
ERROR	106	6.12646631		

Tests of Hypotheses:

a) using the Type III MS for GENO*REP as an error term

Source	DF	Type III SS	F Value	Pr > F
GENO	3	5.97320268	47.99	0.0049

b) using the Type III MS for CUT*REP(GENO) as an error term

Source	DF	Type III SS	F Value	Pr > F
CUT	2	2.18064589	14.65	0.0021

c) using the Type III MS for CUT*REP(GENO) as an error term

Source	DF	Type III SS	F Value	Pr > F
CUT*GENO	6	0.73851151	1.65	0.2491

7.2: Analysis of AP Results for the Cortex

Source	DF	Type III SS	F Value	Pr > F
GENO	3	2.85710198	33.66	0.0001
REP	1	0.07199849	2.54	0.1137
GENO*REP	3	0.09722159	1.15	0.3344
CUT	2	8.36511464	147.83	0.0001
CUT*GENO	6	0.33132704	1.95	0.0792
CUT*REP(GENO)	8	0.15231416	0.67	0.7143
ERROR	106	3.04041666		

Tests of Hypotheses:

a) using the Type III MS for GENO*REP as an error term

Source	DF	Type III SS	F Value	Pr > F
GENO	3	2.85710198	29.39	0.0100

b) using the Type III MS for CUT*REP(GENO) as an error term

Source	DF	Type III SS	F Value	Pr > F
CUT	2	8.36511464	219.68	0.0001

c) using the Type III MS for CUT*REP(GENO) as an error term

Source	DF	Type III SS	F Value	Pr > F
CUT*GENO	6	0.33132704	2.90	0.0830

7.3: Analysis of AP Results for the Sclerenchyma Ring

Source	DF	Type III SS	F Value	Pr > F
GENO	3	1.61299043	19.85	0.0001
REP	1	0.01413612	0.52	0.4717
GENO*REP	3	0.23386723	2.88	0.0396
CUT	2	7.11479843	131.31	0.0001
CUT*GENO	6	0.71386482	4.39	0.0005
CUT*REP(GENO)	8	0.71758890	3.31	0.0021
ERROR	106	2.88135135		

Tests of Hypotheses:

a) using the Type III MS for GENO*REP as an error term

Source	DF	Type III SS	F Value	Pr > F
GENO	3	1.61299043	6.90	0.0735

b) using the Type III MS for CUT*REP(GENO) as an error term

Source	DF	Type III SS	F Value	Pr > F
CUT	2	7.11479843	39.66	0.0001

c) using the Type III MS for CUT*REP(GENO) as an error term

Source	DF	Type III SS	F Value	Pr > F
CUT*GENO	6	0.71386482	1.33	0.3462

7.4: Analysis of AP Results for the Vascular Bundle

Source	DF	Type III SS	F Value	Pr > F
GENO	3	0.73173169	48.91	0.0001
REP	1	0.00200231	0.40	0.5277
GENO*REP	3	0.01672885	1.12	0.3452
CUT	2	0.64628402	64.80	0.0001
CUT*GENO	6	0.03966672	1.33	0.2522
CUT*REP(GENO)	8	0.06469427	1.62	0.1273
ERROR	106	0.52371327		

Tests of Hypotheses:

a) using the Type III MS for GENO*REP as an error term

Source	DF	Type III SS	F Value	Pr > F
GENO	3	0.73173169	43.74	0.0056

b) using the Type III MS for CUT*REP(GENO) as an error term

Source	DF	Type III SS	F Value	Pr > F
CUT	2	0.64628402	39.96	0.0001

c) using the Type III MS for CUT*REP(GENO) as an error term

Source	DF	Type III SS	F Value	Pr > F
CUT*GENO	6	0.03966672	0.82	0.5855

7.5: Analysis of AP Results for the Parenchyma

Source	DF	Type III SS	F Value	Pr > F
GENO	3	1.94783725	27.59	0.0001
REP	1	0.15460250	6.57	0.0118
GENO*REP	3	0.20389264	2.89	0.0391
CUT	2	6.55004065	139.16	0.0001
CUT*GENO	6	0.98445748	6.97	0.0001
CUT*REP(GENO)	8	0.34718690	1.84	0.0769
ERROR	106	2.50719347		

Tests of Hypotheses:

a) using the Type III MS for GENO*REP as an error term

Source	DF	Type III SS	F Value	Pr > F
GENO	3	1.94783725	9.55	0.0481

b) using the Type III MS for CUT*REP(GENO) as an error term

Source	DF	Type III SS	F Value	Pr > F
CUT	2	6.55004065	75.46	0.0001

c) using the Type III MS for CUT*REP(GENO) as an error term

Source	DF	Type III SS	F Value	Pr > F
CUT*GENO	6	0.98445748	3.78	0.0435

7.6: Analysis of CS Results for the Epidermis

Source	DF	Type III SS	F Value	Pr > F
GENO	3	0.10279038	16.41	0.0001
REP	1	0.00613491	2.94	0.0894
GENO*REP	3	0.00633325	1.01	0.3909
CUT	2	0.03504143	8.39	0.0004
CUT*GENO	6	0.09800048	7.82	0.0001
CUT*REP(GENO)	8	0.04178420	2.50	0.0158
ERROR	106	0.22881745		

Tests of Hypotheses:

a) using the Type III MS for GENO*REP as an error term

Source	DF	Type III SS	F Value	Pr > F
GENO	3	0.10279038	16.23	0.0233

b) using the Type III MS for CUT*REP(GENO) as an error term

Source	DF	Type III SS	F Value	Pr > F
CUT	2	0.03504143	3.35	0.0875

c) using the Type III MS for CUT*REP(GENO) as an error term

Source	DF	Type III SS	F Value	Pr > F
CUT*GENO	6	0.09800048	3.13	0.0696

7.7: Analysis of CS Results for the Cortex

Source	DF	Type III SS	F Value	Pr > F
GENO	3	0.11839941	3.54	0.0173
REP	1	0.00330411	0.30	0.5875
GENO*REP	3	0.00888598	0.27	0.8501
CUT	2	0.86826535	38.91	0.0001
CUT*GENO	6	0.20765205	3.10	0.0078
CUT*REP(GENO)	8	0.01334424	0.15	0.9964
ERROR	106	1.18494057		

Tests of Hypotheses:

a) using the Type III MS for GENO*REP as an error term

Source	DF	Type III SS	F Value	Pr > F
GENO	3	0.11839941	13.32	0.0306

b) using the Type III MS for CUT*REP(GENO) as an error term

Source	DF	Type III SS	F Value	Pr > F
CUT	2	0.86826535	260.27	0.0001

c) using the Type III MS for CUT*REP(GENO) as an error term

Source	DF	Type III SS	F Value	Pr > F
CUT*GENO	6	0.20765205	20.75	0.0002

7.8: Analysis of CS Results for the Sclerenchyma Ring

Source	DF	Type III SS	F Value	Pr > F
GENO	3	0.21281204	11.39	0.0001
REP	1	0.00873082	1.40	0.2392
GENO*REP	3	0.00644991	0.35	0.7927
CUT	2	0.49269942	39.54	0.0001
CUT*GENO	6	0.35074209	9.38	0.0001
CUT*REP(GENO)	8	0.03686603	0.74	0.6563
ERROR	106	0.65727877		

Tests of Hypotheses:

a) using the Type III MS for GENO*REP as an error term

Source	DF	Type III SS	F Value	Pr > F
GENO	3	0.21281204	32.99	0.0085

b) using the Type III MS for CUT*REP(GENO) as an error term

Source	DF	Type III SS	F Value	Pr > F
CUT	2	0.49269942	53.46	0.0001

c) using the Type III MS for CUT*REP(GENO) as an error term

Source	DF	Type III SS	F Value	Pr > F
CUT*GENO	6	0.35074209	12.69	0.0010

7.9: Analysis of CS Results for the Vascular Bundle

Source	DF	Type III SS	F Value	Pr > F
GENO	3	0.20865538	18.43	0.0001
REP	1	0.08543640	22.64	0.0001
GENO*REP	3	0.03875232	3.42	0.0199
CUT	2	0.46974780	62.24	0.0001
CUT*GENO	6	0.11963007	5.28	0.0001
CUT*REP(GENO)	8	0.08140138	2.70	0.0097
ERROR	106	0.39627499		

Tests of Hypotheses:

a) using the Type III MS for GENO*REP as an error term

Source	DF	Type III SS	F Value	Pr > F
GENO	3	0.20865538	5.38	0.1001

b) using the Type III MS for CUT*REP(GENO) as an error term

Source	DF	Type III SS	F Value	Pr > F
CUT	2	0.46974780	23.08	0.0005

Tests of Hypotheses using the Type III MS for CUT*REP(GENO) as an error term

Source	DF	Type III SS	F Value	Pr > F
CUT*GENO	6	0.11963007	1.96	0.1861

7.10: Analysis of CS Results for the Parenchyma

Source	DF	Type III SS	F Value	Pr > F
GENO	3	0.16857957	15.42	0.0001
REP	1	0.00000340	0.00	0.9757
GENO*REP	3	0.00470607	0.43	0.7315
CUT	2	0.61181455	83.97	0.0001
CUT*GENO	6	0.22542132	10.31	0.0001
CUT*REP(GENO)	8	0.00535262	0.18	0.9927
ERROR	106	0.40007073		

Tests of Hypotheses:

a) using the Type III MS for GENO*REP as an error term

Source	DF	Type III SS	F Value	Pr > F
GENO	3	0.16857957	35.82	0.0075

b) using the Type III MS for CUT*REP(GENO) as an error term

Source	DF	Type III SS	F Value	Pr > F
CUT	2	0.61181455	457.21	0.0001

c) using the Type III MS for CUT*REP(GENO) as an error term

Source	DF	Type III SS	F Value	Pr > F
CUT*GENO	6	0.22542132	56.15	0.0001

APPENDIX 8.
ANALYSIS OF VARIANCE OF SECTION-TO-SLIDE RESULTS FOR THE PARENCHYMA DISAPPEARANCE IN RCG STEM SECTIONS AFTER 24 HOURS IN RUMEN FLUID.

Source	DF	Type III SS	F Value	Pr > F
GENO	3	0.06623720	2.43	0.0773
REP	1	0.00478236	0.53	0.4719
GENO*REP	3	0.00464357	0.17	0.9159
CUT	2	0.36315093	19.98	0.0001
CUT*GENO	6	0.03630006	0.67	0.6777
CUT*REP(GENO)	8	0.13802638	1.90	0.0833
ERROR	106	0.43517898		

Tests of Hypotheses:

a) using the Type III MS for GENO*REP as an error term

Source	DF	Type III SS	F Value	Pr > F
GENO	3	0.06623720	14.26	0.0279

b) using the Type III MS for CUT*REP(GENO) as an error term

Source	DF	Type III SS	F Value	Pr > F
CUT	2	0.36315093	10.52	0.0058

c) using the Type III MS for CUT*REP(GENO) as an error term

Source	DF	Type III SS	F Value	Pr > F
CUT*GENO	6	0.03630006	0.35	0.8907

APPENDIX 9.
ANALYSIS OF VARIANCE OF UNDIGESTED PERCENT AREA OF TISSUES IN RCG STEM SECTIONS.

9.1: Analysis of the Area of the Epidermis

Source	DF	Type III SS	F Value	Pr > F
GENO	3	0.00143743	1.08	0.3630
REP	1	0.00080185	1.80	0.1827
GENO*REP	3	0.00289316	2.16	0.0967
CUT	2	0.00112319	1.26	0.2879
CUT*GENO	6	0.00222509	0.83	0.5477
CUT*REP(GENO)	8	0.00853926	2.40	0.0205
ERROR	106	0.04686830		

Tests of Hypotheses:

a) using the Type III MS for GENO*REP as an error term

Source	DF	Type III SS	F Value	Pr > F
GENO	3	0.00143743	0.50	0.7099

b) using the Type III MS for CUT*REP(GENO) as an error term

Source	DF	Type III SS	F Value	Pr > F
CUT	2	0.00112319	0.53	0.6100

c) using the Type III MS for CUT*REP(GENO) as an error term

Source	DF	Type III SS	F Value	Pr > F
CUT*GENO	6	0.00222509	0.35	0.8927

9.2: Analysis of the Area of the Cortex

Source	DF	Type III SS	F Value	Pr > F
GENO	3	0.01309689	2.06	0.1099
REP	1	0.00144501	0.68	0.4107
GENO*REP	3	0.00772876	1.22	0.3075
CUT	2	0.00322125	0.76	0.4700
CUT*GENO	6	0.02345458	1.85	0.0973
CUT*REP(GENO)	8	0.00982531	0.58	0.7925
ERROR	106	0.22447780		

Tests of Hypotheses:

a) using the Type III MS for GENO*REP as an error term

Source	DF	Type III SS	F Value	Pr > F
GENO	3	0.01309689	1.69	0.3377

b) using the Type III MS for CUT*REP(GENO) as an error term

Source	DF	Type III SS	F Value	Pr > F
CUT	2	0.00322125	1.31	0.3217

c) using the Type III MS for CUT*REP(GENO) as an error term

Source	DF	Type III SS	F Value	Pr > F
CUT*GENO	6	0.02345458	3.18	0.0667

9.3: Analysis of the Area of the Phloem

Source	DF	Type III SS	F Value	Pr > F
GENO	3	0.00682058	14.25	0.0001
REP	1	0.00170707	10.70	0.0015
GENO*REP	3	0.00012578	0.26	0.8521
CUT	2	0.00081614	2.56	0.0823
CUT*GENO	6	0.00058497	0.61	0.7210
CUT*REP(GENO)	8	0.00154939	1.21	0.2981
ERROR	106	0.01676869		

Tests of Hypotheses:

a) using the Type III MS for GENO*REP as an error term

Source	DF	Type III SS	F Value	Pr > F
GENO	3	0.00682058	54.23	0.0041

b) using the Type III MS for CUT*REP(GENO) as an error term

Source	DF	Type III SS	F Value	Pr > F
CUT	2	0.00081614	2.11	0.1840

c) using the Type III MS for CUT*REP(GENO) as an error term

Source	DF	Type III SS	F Value	Pr > F
CUT*GENO	6	0.00058497	0.50	0.7907

9.4: Analysis of the Area of the Sclerenchyma Ring

Source	DF	Type III SS	F Value	Pr > F
GENO	3	0.03968278	7.84	0.0001
REP	1	0.00050178	0.30	0.5866
GENO*REP	3	0.00306522	0.61	0.6127
CUT	2	0.02309118	6.85	0.0016
CUT*GENO	6	0.01573023	1.55	0.1678
CUT*REP(GENO)	8	0.01729369	1.28	0.2609
ERROR	106	0.18121886		

Tests of Hypotheses:

a) using the Type III MS for GENO*REP as an error term

Source	DF	Type III SS	F Value	Pr > F
GENO	3	0.03968278	12.95	0.0319

b) using the Type III MS for CUT*REP(GENO) as an error term

Source	DF	Type III SS	F Value	Pr > F
CUT	2	0.02309118	5.34	0.0336

c) using the Type III MS for CUT*REP(GENO) as an error term

Source	DF	Type III SS	F Value	Pr > F
CUT*GENO	6	0.01573023	1.21	0.3891

9.5: Analysis of the Area of the Xylem

Source	DF	Type III SS	F Value	Pr > F
GENO	3	0.00118112	2.42	0.0703
REP	1	0.00017483	1.07	0.3023
GENO*REP	3	0.00029337	0.60	0.6158
CUT	2	0.00047301	1.45	0.2384
CUT*GENO	6	0.00081357	0.83	0.5468
CUT*REP(GENO)	8	0.00240472	1.85	0.0764
ERROR	106	0.01708907		

Tests of Hypotheses:

a) using the Type III MS for GENO*REP as an error term

Source	DF	Type III SS	F Value	Pr > F
GENO	3	0.00118112	4.03	0.1413

b) using the Type III MS for CUT*REP(GENO) as an error term

Source	DF	Type III SS	F Value	Pr > F
CUT	2	0.00047301	0.79	0.4876

c) using the Type III MS for CUT*REP(GENO) as an error term

Source	DF	Type III SS	F Value	Pr > F
CUT*GENO	6	0.00081357	0.45	0.8261

9.6: Analysis of the Area of the Vascular Bundle

Source	DF	Type III SS	F Value	Pr > F
GENO	3	0.00518990	2.43	0.0695
REP	1	0.00000766	0.01	0.9176
GENO*REP	3	0.00216580	1.01	0.3900
CUT	2	0.00105690	0.74	0.4788
CUT*GENO	6	0.00162915	0.38	0.8897
CUT*REP(GENO)	8	0.00609689	1.07	0.3901
ERROR	106	0.07482539		

Tests of Hypotheses:

a) using the Type III MS for GENO*REP as an error term

Source	DF	Type III SS	F Value	Pr > F
GENO	3	0.00518990	2.40	0.2458

b) using the Type III MS for CUT*REP(GENO) as an error term

Source	DF	Type III SS	F Value	Pr > F
CUT	2	0.00105690	0.69	0.5276

c) using the Type III MS for CUT*REP(GENO) as an error term

Source	DF	Type III SS	F Value	Pr > F
CUT*GENO	6	0.00162915	0.36	0.8873

9.7: Analysis of the Area of the Parenchyma

Source	DF	Type III SS	F Value	Pr > F
GENO	3	0.04804717	7.80	0.0001
REP	1	0.00037229	0.18	0.6711
GENO*REP	3	0.00337698	0.55	0.6504
CUT	2	0.00943497	2.30	0.1055
CUT*GENO	6	0.02619707	2.13	0.0563
CUT*REP(GENO)	8	0.02920968	1.78	0.0895
ERROR	106	0.22160580		

Tests of Hypotheses:

a) using the Type III MS for GENO*REP as an error term

Source	DF	Type III SS	F Value	Pr > F
GENO	3	0.04804717	14.23	0.0280

b) using the Type III MS for CUT*REP(GENO) as an error term

Source	DF	Type III SS	F Value	Pr > F
CUT	2	0.00943497	1.29	0.3264

c) using the Type III MS for CUT*REP(GENO) as an error term

Source	DF	Type III SS	F Value	Pr > F
CUT*GENO	6	0.02619707	1.20	0.3960

APPENDIX 10.
ANALYSIS OF VARIANCE OF CELL WALL LENGTH OF INDIVIDUAL TISSUES IN RCG STEM SECTIONS.

10.1: Analysis of the Length of the Epidermis

Source	DF	Type III SS	F Value	Pr > F
GENO	3	14756.34189	2.55	0.0599
REP	1	913.12146	0.47	0.4933
GENO*REP	3	4879.18418	0.84	0.4739
CUT	2	2182.19943	0.56	0.5702
CUT*GENO	6	24304.89669	2.10	0.0596
CUT*REP(GENO)	8	40144.65314	2.60	0.0124
ERROR	106	204027.93620		

Tests of Hypotheses:

a) using the Type III MS for GENO*REP as an error term

Source	DF	Type III SS	F Value	Pr > F
GENO	3	14756.34189	3.02	0.1938

b) using the Type III MS for CUT*REP(GENO) as an error term

Source	DF	Type III SS	F Value	Pr > F
CUT	2	2182.199431	0.22	0.8092

c) using the Type III MS for CUT*REP(GENO) as an error term

Source	DF	Type III SS	F Value	Pr > F
CUT*GENO	6	24304.89669	0.81	0.5917

10.2: Analysis of the Length of the Cortex

Source	DF	Type III SS	F Value	Pr > F
GENO	3	944.103575	2.34	0.0781
REP	1	66.627765	0.49	0.4835
GENO*REP	3	1150.073201	2.84	0.0412
CUT	2	18.269587	0.07	0.9345
CUT*GENO	6	1938.227162	2.40	0.0328
CUT*REP(GENO)	8	1195.262062	1.11	0.3634
ERROR	106	14228.094920		

Tests of Hypotheses:

a) using the Type III MS for GENO*REP as an error term

Source	DF	Type III SS	F Value	Pr > F
GENO	3	944.1035753	0.82	0.5625

b) using the Type III MS for CUT*REP(GENO) as an error term

Source	DF	Type III SS	F Value	Pr > F
CUT	2	18.26958733	0.06	0.9411

c) using the Type III MS for CUT*REP(GENO) as an error term

Source	DF	Type III SS	F Value	Pr > F
CUT*GENO	6	1938.227162	2.16	0.1546

10.3: Analysis of the Length of the Phloem

Source	DF	Type III SS	F Value	Pr > F
GENO	3	3276610.62	0.62	0.6060
REP	1	6351164.04	3.58	0.0611
GENO*REP	3	2572712.72	0.48	0.6942
CUT	2	15970284.22	4.51	0.0133
CUT*GENO	6	4812424.21	0.45	0.8417
CUT*REP(GENO)	8	24428204.78	1.72	0.1015
ERROR	106	191353462.70		

Tests of Hypotheses:

a) using the Type III MS for GENO*REP as an error term

Source	DF	Type III SS	F Value	Pr > F
GENO	3	3276610.616	1.27	0.4236

b) using the Type III MS for CUT*REP(GENO) as an error term

Source	DF	Type III SS	F Value	Pr > F
CUT	2	15970284.22	2.62	0.1337

c) using the Type III MS for CUT*REP(GENO) as an error term

Source	DF	Type III SS	F Value	Pr > F
CUT*GENO	6	4812424.206	0.26	0.9397

10.4: Analysis of the Length of the Sclerenchyma Ring

Source	DF	Type III SS	F Value	Pr > F
GENO	3	41.56214263	4.50	0.0052
REP	1	0.00855939	0.00	0.9580
GENO*REP	3	24.23561867	2.62	0.0543
CUT	2	43.27967922	7.03	0.0014
CUT*GENO	6	11.28445613	0.61	0.7209
CUT*REP(GENO)	8	7.47265699	0.30	0.9632
ERROR	106	326.51794500		

Tests of Hypotheses:

a) using the Type III MS for GENO*REP as an error term

Source	DF	Type III SS	F Value	Pr > F
GENO	3	41.56214263	1.71	0.3343

b) using the Type III MS for CUT*REP(GENO) as an error term

Source	DF	Type III SS	F Value	Pr > F
CUT	2	43.27967922	23.17	0.0005

c) using the Type III MS for CUT*REP(GENO) as an error term

Source	DF	Type III SS	F Value	Pr > F
CUT*GENO	6	11.28445613	2.01	0.1770

10.5: Analysis of the Length of the Xylem

Source	DF	Type III SS	F Value	Pr > F
GENO	3	2.85670E+10	0.56	0.6405
REP	1	2.38939E+10	1.41	0.2372
GENO*REP	3	2.90767E+10	0.57	0.6339
CUT	2	2.06792E+10	0.61	0.5444
CUT*GENO	6	6.91720E+10	0.68	0.6646
CUT*REP(GENO)	8	1.27960E+11	0.95	0.4824
ERROR	106	1.77877E+12		

Tests of Hypotheses:

a) using the Type III MS for GENO*REP as an error term

Source	DF	Type III SS	F Value	Pr > F
GENO	3	28567015002	0.98	0.5056

b) using the Type III MS for CUT*REP(GENO) as an error term

Source	DF	Type III SS	F Value	Pr > F
CUT	2	20679160966	0.65	0.5492

c) using the Type III MS for CUT*REP(GENO) as an error term

Source	DF	Type III SS	F Value	Pr > F
CUT*GENO	6	69171994253	0.72	0.6453

10.6: Analysis of the Length of the Vascular Bundle

Source	DF	Type III SS	F Value	Pr > F
GENO	3	80.8228731	0.93	0.4269
REP	1	135.2124967	4.69	0.0326
GENO*REP	3	120.3520906	1.39	0.2495
CUT	2	61.7599437	1.07	0.3464
CUT*GENO	6	215.1926727	1.24	0.2900
CUT*REP(GENO)	8	239.6810424	1.04	0.4118
ERROR	106	3039.2988450		

Tests of Hypotheses:

a) using the Type III MS for GENO*REP as an error term

Source	DF	Type III SS	F Value	Pr > F
GENO	3	80.82287310	0.67	0.6243

b) using the Type III MS for CUT*REP(GENO) as an error term

Source	DF	Type III SS	F Value	Pr > F
CUT	2	61.75994366	1.03	0.3997

Tests of Hypotheses using the Type III MS for CUT*REP(GENO) as an error term

Source	DF	Type III SS	F Value	Pr > F
CUT*GENO	6	215.1926727	1.20	0.3955

10.7: Analysis of the Length of the Parenchyma

Source	DF	Type III SS	F Value	Pr > F
GENO	3	0.01535816	1.08	0.3610
REP	1	0.00004022	0.01	0.9268
GENO*REP	3	0.04267867	3.00	0.0339
CUT	2	0.02395098	2.53	0.0848
CUT*GENO	6	0.04823530	1.70	0.1292
CUT*REP(GENO)	8	0.07904076	2.08	0.0437
PLANT	1	0.00498626	1.05	0.3074
ERROR	105	0.50273969		

Tests of Hypotheses:

a) using the Type III MS for GENO*REP as an error term

Source	DF	Type III SS	F Value	Pr > F
GENO	3	0.01535816	0.36	0.7882

b) using the Type III MS for CUT*REP(GENO) as an error term

Source	DF	Type III SS	F Value	Pr > F
CUT	2	0.02395098	1.21	0.3469

c) using the Type III MS for CUT*REP(GENO) as an error term

Source	DF	Type III SS	F Value	Pr > F
CUT*GENO	6	0.04823530	0.81	0.5878

APPENDIX 11.
ANALYSIS OF VARIANCE OF CELL WALL WIDTH OF INDIVIDUAL TISSUES IN RCG STEM SECTIONS.

11.1: Analysis of the Width of the Epidermis

Source	DF	Type III SS	F Value	Pr > F
GENO	3	0.01885451	5.72	0.0012
REP	1	0.00048572	0.44	0.5077
GENO*REP	3	0.00426672	1.29	0.2806
CUT	2	0.00743427	3.38	0.0378
CUT*GENO	6	0.01931949	2.93	0.0111
CUT*REP(GENO)	8	0.01907807	2.17	0.0357
ERROR	106	0.11626207		

Tests of Hypotheses:

a) using the Type III MS for GENO*REP as an error term

Source	DF	Type III SS	F Value	Pr > F
GENO	3	0.01885451	4.42	0.1269

b) using the Type III MS for CUT*REP(GENO) as an error term

Source	DF	Type III SS	F Value	Pr > F
CUT	2	0.00743427	1.56	0.2681

c) using the Type III MS for CUT*REP(GENO) as an error term

Source	DF	Type III SS	F Value	Pr > F
CUT*GENO	6	0.01931949	1.35	0.3379

11.2: Analysis of the Width of the Cortex

Source	DF	Type III SS	F Value	Pr > F
GENO	3	0.01119610	0.44	0.7267
REP	1	0.00416369	0.49	0.4864
GENO*REP	3	0.01958706	0.77	0.5161
CUT	2	0.01345844	0.79	0.4571
CUT*GENO	6	0.05203083	1.02	0.4188
CUT*REP(GENO)	8	0.04228894	0.62	0.7598
ERROR	106	0.92082243		

Tests of Hypotheses:

a) using the Type III MS for GENO*REP as an error term

Source	DF	Type III SS	F Value	Pr > F
GENO	3	0.01119610	0.57	0.6714

b) using the Type III MS for CUT*REP(GENO) as an error term

Source	DF	Type III SS	F Value	Pr > F
CUT	2	0.01345844	1.27	0.3311

c) using the Type III MS for CUT*REP(GENO) as an error term

Source	DF	Type III SS	F Value	Pr > F
CUT*GENO	6	0.05203083	1.64	0.2526

11.3: Analysis of the Width of the Phloem

Source	DF	Type III SS	F Value	Pr > F
GENO	3	0.00059794	0.10	0.9603
REP	1	0.01605374	7.99	0.0056
GENO*REP	3	0.02665934	4.42	0.0057
CUT	2	0.00477114	1.19	0.3092
CUT*GENO	6	0.00934836	0.78	0.5911
CUT*REP(GENO)	8	0.01882050	1.17	0.3239
ERROR	106	0.21219211		

Tests of Hypotheses:

a) using the Type III MS for GENO*REP as an error term

Source	DF	Type III SS	F Value	Pr > F
GENO	3	0.00059794	0.02	0.9945

b) using the Type III MS for CUT*REP(GENO) as an error term

Source	DF	Type III SS	F Value	Pr > F
CUT	2	0.00477114	1.01	0.4050

c) using the Type III MS for CUT*REP(GENO) as an error term

Source	DF	Type III SS	F Value	Pr > F
CUT*GENO	6	0.00934836	0.66	0.6833

11.4: Analysis of the Width of the Sclerenchyma Ring

Source	DF	Type III SS	F Value	Pr > F
GENO	3	0.00451944	2.09	0.1055
REP	1	0.00140763	1.96	0.1649
GENO*REP	3	0.00180239	0.84	0.4776
CUT	2	0.00266236	1.85	0.1623
CUT*GENO	6	0.00572490	1.33	0.2521
CUT*REP(GENO)	8	0.00648111	1.13	0.3521
ERROR	106	0.07555656		

Tests of Hypotheses:

a) using the Type III MS for GENO*REP as an error term

Source	DF	Type III SS	F Value	Pr > F
GENO	3	0.00451944	2.51	0.2351

b) using the Type III MS for CUT*REP(GENO) as an error term

Source	DF	Type III SS	F Value	Pr > F
CUT	2	0.00266236	1.64	0.2524

c) using the Type III MS for CUT*REP(GENO) as an error term

Source	DF	Type III SS	F Value	Pr > F
CUT*GENO	6	0.00572490	1.18	0.4035

11.5: Analysis of the Width of the Xylem

Source	DF	Type III SS	F Value	Pr > F
GENO	3	0.01126660	1.27	0.2889
REP	1	0.00009406	0.03	0.8589
GENO*REP	3	0.00170069	0.19	0.9020
CUT	2	0.02084056	3.52	0.0331
CUT*GENO	6	0.02636482	1.48	0.1905
CUT*REP(GENO)	8	0.03124075	1.32	0.2419
ERROR	106	0.31085291		

Tests of Hypotheses:

a) using the Type III MS for GENO*REP as an error term

Source	DF	Type III SS	F Value	Pr > F
GENO	3	0.01126660	6.62	0.0774

b) using the Type III MS for CUT*REP(GENO) as an error term

Source	DF	Type III SS	F Value	Pr > F
CUT	2	0.02084056	2.67	0.1295

c) using the Type III MS for CUT*REP(GENO) as an error term

Source	DF	Type III SS	F Value	Pr > F
CUT*GENO	6	0.02636482	1.13	0.4261

11.6: Analysis of the Width of the Vascular Bundle

Source	DF	Type III SS	F Value	Pr > F
GENO	3	0.00816979	3.20	0.0263
REP	1	0.00353583	4.16	0.0439
GENO*REP	3	0.00578896	2.27	0.0847
CUT	2	0.00213653	1.26	0.2888
CUT*GENO	6	0.00549869	1.08	0.3804
CUT*REP(GENO)	8	0.01602064	2.36	0.0226
ERROR	106	0.08931009		

Tests of Hypotheses:

a) using the Type III MS for GENO*REP as an error term

Source	DF	Type III SS	F Value	Pr > F
GENO	3	0.00816979	1.41	0.3919

b) using the Type III MS for CUT*REP(GENO) as an error term

Source	DF	Type III SS	F Value	Pr > F
CUT	2	0.00213653	0.53	0.6061

c) using the Type III MS for CUT*REP(GENO) as an error term

Source	DF	Type III SS	F Value	Pr > F
CUT*GENO	6	0.00549869	0.46	0.8217

11.7: Analysis of the Width of the Parenchyma

Source	DF	Type III SS	F Value	Pr > F
GENO	3	0.00191863	1.30	0.2778
REP	1	0.00000031	0.00	0.9801
GENO*REP	3	0.00190534	1.29	0.2808
CUT	2	0.00523236	5.33	0.0063
CUT*GENO	6	0.00555273	1.88	0.0903
CUT*REP(GENO)	8	0.00930453	2.37	0.0220
ERROR	106	0.05203392		

Tests of Hypotheses:

a) using the Type III MS for GENO*REP as an error term

Source	DF	Type III SS	F Value	Pr > F
GENO	3	0.00191863	1.01	0.4978

b) using the Type III MS for CUT*REP(GENO) as an error term

Source	DF	Type III SS	F Value	Pr > F
CUT	2	0.00523236	2.25	0.1678

Tests of Hypotheses using the Type III MS for CUT*REP(GENO) as an error term

Source	DF	Type III SS	F Value	Pr > F
CUT*GENO	6	0.00555273	0.80	0.5986

APPENDIX 12.
ANALYSIS OF VARIANCE OF VASCULAR BUNDLE NUMBER IN RCG STEM SECTIONS.

Source	DF	Type III SS	F Value	Pr > F
GENO	3	0.29931199	4.01	0.0095
REP	1	0.00264312	0.11	0.7450
GENO*REP	3	0.31995153	4.29	0.0067
CUT	2	0.01051817	0.21	0.8097
CUT*GENO	6	0.36521165	2.45	0.0295
CUT*REP(GENO)	8	0.25359212	1.28	0.2643
ERROR	106	2.61635508		

Tests of Hypotheses:

a) using the Type III MS for GENO*REP as an error term

Source	DF	Type III SS	F Value	Pr > F
GENO	3	0.29931199	0.94	0.5212

b) using the Type III MS for CUT*REP(GENO) as an error term

Source	DF	Type III SS	F Value	Pr > F
CUT	2	0.01051817	0.17	0.8500

c) using the Type III MS for CUT*REP(GENO) as an error term

Source	DF	Type III SS	F Value	Pr > F
CUT*GENO	6	0.36521165	1.92	0.1930

APPENDIX 13.
ANALYSIS OF VARIANCE OF DRY MATTER DIGESTIBILITY OF RCG STEMS USING A PREPARED CELLULASE INCUBATION FOR 16 HOURS.

Source	DF	Type III SS	F Value	Pr > F
GENO	3	18.6381250	5.23	0.0105
REP	1	43.2306250	36.37	0.0001
GENO*REP	3	69.8781250	19.59	0.0001
CUT	2	37.1340000	15.62	0.0002
CUT*GENO	6	116.9510000	16.40	0.0001
CUT*REP(GENO)	8	41.3190000	4.34	0.0060
ERROR	16	19.0200000		

Tests of Hypotheses:

a) using the Type III MS for GENO*REP as an error term

Source	DF	Type III SS	F Value	Pr > F
GENO	3	18.63812500	0.27	0.8468

b) using the Type III MS for CUT*REP(GENO) as an error term

Source	DF	Type III SS	F Value	Pr > F
CUT	2	37.13400000	3.59	0.0769

c) using the Type III MS for CUT*REP(GENO) as an error term

Source	DF	Type III SS	F Value	Pr > F
CUT*GENO	6	116.9510000	3.77	0.0437

APPENDIX 14.
ANALYSIS OF VARIANCE FOR NEUTRAL SUGARS RELEASED DURING A 16 HOUR CELLULASE INCUBATION OF RCG STEMS.

14.1: Analysis of Fructose

Source	DF	Type III SS	F Value	Pr > F
GENO	3	11.93805000	1.91	0.3043
REP	1	0.57245000	0.27	0.6365
ERROR	3	6.25245000		

14.2: Analysis of Xylose

Source	DF	Type III SS	F Value	Pr > F
GENO	3	7.74905000	0.51	0.7020
REP	1	0.26645000	0.05	0.8331
ERROR	3	15.14285000		

14.3: Analysis of Glucose

Source	DF	Type III SS	F Value	Pr > F
GENO	3	40.58495000	0.99	0.5043
REP	1	16.41645000	1.20	0.3539
ERROR	3	41.14335000		

14.4: Analysis of Galactose

Source	DF	Type III SS	F Value	Pr > F
GENO	3	0.02690000	0.09	0.9585
REP	1	0.05780000	0.60	0.4934
ERROR	3	0.28670000		

14.5: Analysis of Arabinose

Source	DF	Type III SS	F Value	Pr > F
GENO	3	1.69393750	4.98	0.1101
REP	1	0.62161250	5.48	0.1011
ERROR	3	0.34023750		

APPENDIX 15.
ANALYSIS OF VARIANCE OF PHENOLIC ACIDS RELEASED DURING A 16 HOUR CELLULASE INCUBATION OF RCG STEMS.

15.1: Analysis of p-Coumaric Acid

Source	DF	Type III SS	F Value	Pr > F
GENO	3	0.00573750	1.77	0.3250
REP	1	0.00151250	1.40	0.3217
ERROR	3	0.00323750		

15.1: Analysis of Ferulic Acid

Source	DF	Type III SS	F Value	Pr > F
GENO	3	0.02033750	1.03	0.4921
REP	1	0.00011250	0.02	0.9045
ERROR	3	0.01983750		

15.1: Analysis of the Ratio of FA:PCA

Source	DF	Type III SS	F Value	Pr > F
GENO	3	0.09053750	2.63	0.2241
REP	1	0.00911250	0.79	0.4386
ERROR	3	0.03443750		

APPENDIX 16.
ANALYSIS OF VARIANCE OF NEUTRAL SUGARS RELEASED BY ACID HYDROLYSIS OF RCG STEMS.

16.1: Analysis of Arabinose

Source	DF	Type III SS	F Value	Pr > F
GENO	3	8.44945000	0.78	0.5791
REP	1	3.53780000	0.98	0.3956
ERROR	3	10.85450000		

16.2: Analysis of Galactose

Source	DF	Type III SS	F Value	Pr > F
GENO	3	1.83985000	0.34	0.8015
REP	1	1.71125000	0.94	0.4030
ERROR	3	5.44005000		

16.3: Analysis of Glucose

Source	DF	Type III SS	F Value	Pr > F
GENO	3	67.94550000	0.29	0.8308
REP	1	69.14880000	0.89	0.4150
ERROR	3	233.02170000		

16.4: Analysis of Xylose

Source	DF	Type III SS	F Value	Pr > F
GENO	3	13.66630000	0.18	0.9010
REP	1	0.00245000	0.00	0.9927
ERROR	3	74.29545000		

16.5: Analysis of Fructose

Source	DF	Type III SS	F Value	Pr > F
GENO	3	44.30943750	0.96	0.5128
REP	1	0.00551250	0.00	0.9861
ERROR	3	46.13073750		

APPENDIX 17.
ANALYSIS OF VARIANCE OF PHENOLIC ACIDS RELEASED BY ALKALINE HYDROLYSIS OF RCG STEMS.

17.1: Analysis of p-Courmaric Acid

Source	DF	Type III SS	F Value	Pr > F
GENO	3	0.08583750	6.29	0.0825
REP	1	0.00551250	1.21	0.3512
ERROR	3	0.01363750		

17.2: Analysis of Ferulic Acid

Source	DF	Type III SS	F Value	Pr > F
GENO	3	0.02383750	0.82	0.5636
REP	1	0.00101250	0.10	0.7680
ERROR	3	0.02913750		

17.3: Analysis of PCA:FA

Source	DF	Type III SS	F Value	Pr > F
GENO	3	0.00163750	0.48	0.7210
REP	1	0.00001250	0.01	0.9234
ERROR	3	0.00343750		